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**ELECTROPHYSIOLOGY OF
POTASSIUM CHANNELS
IN THE HAMSTER EGG.**

by

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Presented for the degree of

Ph.D.

of the

University of Edinburgh

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This thesis is dedicated to my mother;
I will always be grateful for her support.

Life isn't all beer and skittles.

Thomas Hughes.

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The rest of the thesis is my own work.

SUMMARY

Ca^{2+} -activated K^+ currents were recorded from unfertilised and fertilised golden hamster eggs, using conventional intracellular recording techniques. Activation of the currents was accomplished by various treatments producing a rise in the intracellular free calcium concentration ($[\text{Ca}^{2+}]_i$). The resulting hyperpolarisations of the membrane were investigated using Ca^{2+} extrusion blockers such as La^{3+} , quercetin, 2,4-Dinitrophenol, high pH_o solution and Na^+ -free solution, to alter the time-course of the hyperpolarisations.

Iontophoretic injection of Ca^{2+} and Sr^{2+} produced a transient hyperpolarisation of the hamster egg membrane accompanied by an increase in membrane conductance, mediated by a Ca^{2+} -activated K^+ current. The effects of La^{3+} , quercetin, high pH_o solution and Na^+ -free solution in prolonging the hyperpolarisation suggest that both a Na^+ - Ca^{2+} exchange system and an active Ca^{2+} pump are responsible for the recovery phase of the Ca^{2+} -evoked hyperpolarisations.

A combination of high pH_o and high $[\text{Ca}^{2+}]_o$ solution was found to evoke a sustained hyperpolarisation of the membrane, with an estimated value of the reversal potential of about -85 mV. The membrane potential changed linearly with $\log [\text{K}^+]_o$ with a slope of 43 ± 2 mV (mean \pm S.D., $n=4$) for a 10-fold change in $[\text{K}^+]_o$, while it was unaltered by the removal of Cl^- from the solution. The amplitude of the pH_o -induced hyperpolarisation decreased substantially when $[\text{Ca}^{2+}]_o$ was lowered from 20 to 1 mM and with addition of TEA. Injection of EGTA into the egg prevented the pH_o -induced hyperpolarisation, suggesting that a rise in $[\text{Ca}^{2+}]_i$ is required. The high pH_o does not produce a large increase in pH_i and the onset of the response is rapid, compatible with an external site of action.

A transient hyperpolarisation and increase in conductance could be evoked after a long latency (ca. 9 s) by a single Ca^{2+} action potential in unfertilised hamster eggs. The estimated reversal potential was close to E_K . A second action potential elicited soon after the first did not induce a similar response. A number of treatments (insertion of a Ca^{2+} micro-pipette, application

of Na^+ -free solution, La^{3+} or high pH_o) likely to raise $[\text{Ca}^{2+}]_i$ also induced similar large hyperpolarisations, after which a single Ca^{2+} action potential failed to evoke a large delayed hyperpolarisation. This suggests that a small rise in $[\text{Ca}^{2+}]_i$ activates a slow process leading to a further large increase in $[\text{Ca}^{2+}]_i$.

The fertilisation potential of hamster eggs, consisting of transient, repetitive hyperpolarisations was recorded using intracellular recording techniques. The duration of hyperpolarisations were prolonged by La^{3+} , high pH_o and Na^+ -free solution. Low pH_o decreased the duration. The frequency of the hyperpolarisations was also reduced by Co^{2+} and La^{3+} .

The first single channel recordings from the hamster egg were made, using the excised inside-out patch conformation. Evidence of a channel activated by intracellular Ca^{2+} was obtained. This channel had a reversal potential at 0 mV in symmetrical KCl, and displayed outward rectification over the potential range - 60 - + 60 mV. The conductance was 65.3 ± 12.9 pS at depolarised potentials and 23.4 ± 11.8 pS ($n=4$) at hyperpolarised potentials.

SECTION 1

LITERATURE REVIEW

This review serves as a general introduction to the literature upon which the ideas and results presented in this thesis are based. It is composed of three sections; the first discusses the activation of ion channels in fertilisation and the involvement of Ca^{2+} in this, the second is on the inhibition of Ca^{2+} extrusion, and the third is a resume of the Ca^{2+} -activated K^+ channel in other preparations.

1.1 The egg as a model for electrophysiological investigation of ion channels.

The plasma membrane of the egg was not thought to be electrically excitable until the discovery by Takahashi, Miyazaki and Kidokoro (1971) of an action potential ascribed to Ca^{2+} and Na^+ current in unfertilised Tunicate eggs. Previous to this finding, electrical events induced by sperm activation of the egg, known as the fertilisation potential, had been recorded from a variety of species (see Hagiwara and Jaffe, 1979 for review). These characteristic fertilisation potentials, coupled with the discovery of electrical excitability as an inherent property of most eggs, led to the adoption of the egg as a model for investigation of ion channels. In these early experiments, the techniques used were conventional intracellular recording and two-electrode voltage-clamp recording. Eggs are suitable preparations for the voltage-clamp technique because their spherical shape improves space-clamping.

The first recording of a change in transmembrane potential at fertilisation was obtained from a starfish egg (Tyler, Monroy, Kao and Grundfest, 1956). However, the most thorough investigations of the activation of ionic currents during fertilisation have been performed on sea urchin eggs. After insemination, the membrane potential of the sea urchin egg depolarises rapidly (0.1 to 1 s), from its resting value of about -70 mV, to a peak at about +20 mV. After about a minute, the potential slowly hyperpolarises, returning to its resting value of about -70 mV after 10 minutes or so (Jaffe, 1976). There are at least three ionic species involved in the generation of the fertilisation potential of the sea urchin egg; Na^+ , Ca^{2+} and K^+ . The initial depolarisation was demonstrated to be dependent on Na^+ and in addition to this, the depolarisation leads to the opening of voltage-dependent Ca^{2+} channels (Chambers and de Armendi, 1978). These findings are consistent with results obtained using radioactive tracers, showing that an increase in the uptake of Na^+ (Johnson, Epel and Paul, 1976) and Ca^{2+} (Paul and Johnson, 1978) occurs at fertilisation. The recovery of the membrane potential after this Na^+ and Ca^{2+} -dependent depolarisation is associated with a large increase in K^+ permeability. An eight-fold increase in membrane K^+ conductance,

relative to the unfertilised egg, has been reported after fertilisation of sea urchin eggs (Jaffe and Robinson, 1978). Depolarising fertilisation potentials have also been recorded from eggs of other marine orders such as echinurans (Jaffe, Gould-Somero and Holland, 1979) and annelids (Kline, Jaffe and Tucker, 1985). Once again, Na^+ and Ca^{2+} are the ions underlying these fertilisation potentials. In contrast to the fertilisation potentials of these species, the depolarising phase of the fertilisation potential of amphibian eggs is mediated by an efflux of Cl^- from the egg (Ito, 1972).

Mammalian fertilisation potentials have also been recorded, mainly in the hamster and mouse. In the hamster, transient repetitive hyperpolarisations are recorded after ^{fertilisation} insemination (Miyazaki and Igusa, 1981). These continue for several hours after initiation, superimposed on a slow hyperpolarisation of the membrane potential.

The first report of an electrical event during fertilisation in mouse eggs described a single, small oscillation of the membrane potential of about 4 mV amplitude (Jaffe, Sharp and Wolf, 1983) occurring about 7 min after insemination. Following this, Igusa, Miyazaki and Yamashita (1983) demonstrated that the fertilisation potential of mouse eggs consisted of a series of small, transient hyperpolarisations of the membrane. The reason for the discrepancy in these two reports is probably that the sensitivity of the chart recorder used by Jaffe et al. (1983) was too low; the amplitude of the transient hyperpolarisations in the mouse (about 3-4 mV) is much smaller than in the hamster (about 30-40 mV), making them more difficult to observe.

In both mouse and hamster eggs, it has been shown that the hyperpolarising responses are mediated by a rise in the membrane K^+ conductance. The reversal potential of the responses is about -80 mV, compatible with the equilibrium potential for K^+ or Cl^- and altering the extracellular $[\text{Cl}^-]$ does not abolish the hyperpolarising responses (Miyazaki and Igusa, 1982; Igusa, Miyazaki and Yamashita, 1983). No obvious depolarisation was reported in mammalian eggs prior to these hyperpolarisations, unlike the fertilisation potentials in eggs of lower order animals.

A rise in $[\text{Ca}^{2+}]_i$ occurs at fertilisation.

Despite the different ionic dependencies and characteristic potential changes during fertilisation in different species, one factor is common to all; a rise in the intracellular free $[\text{Ca}^{2+}]$ occurs after insemination. This has been determined in a number of ways; using the Ca^{2+} -sensitive photoprotein aequorin and other Ca^{2+} -sensitive dyes or intracellular Ca^{2+} -sensitive microelectrodes. For example, using aequorin-loaded sea urchin eggs, Steinhardt, Zucker and Schatten (1977) demonstrated a transient rise in intracellular free Ca^{2+} at fertilisation. The amount of light emitted by aequorin depends upon the concentration of Ca^{2+} in the physiological range; the light emission was consistent with a peak Ca^{2+} concentration of about 2.5-4.5 μM , assuming a uniform rise of Ca^{2+} throughout the egg. It reached this peak within one minute and fell to below detectable levels

again within another minute. A value of 2 μM was obtained using the Ca^{2+} -sensitive fluorescent probe Fura-2 (Poenie, Alderton, Tsien and Steinhardt, 1985).

The ability of various parthenogenetic agents to produce a rise in $[\text{Ca}^{2+}]_i$ indicated the involvement of such a rise at fertilisation. For example, parthenogenetic activation of the sea urchin egg had been reported using the Ca^{2+} ionophore A23187 (Steinhardt and Epel, 1974), and a similar rise in Ca^{2+} to that seen at fertilisation was also observed after application of A23187 to aequorin-loaded eggs (Steinhardt et al., 1977). Interestingly, the rise in Ca^{2+} in both fertilised and parthenogenetically activated eggs was unchanged by removing Ca^{2+} from the extracellular solution. This would suggest that the rise in $[\text{Ca}^{2+}]$ produced by these stimuli is due to a release of Ca^{2+} from intracellular stores. A transient increase in Ca^{2+} has similarly been reported in fish eggs (Ridgway, Gilkey and Jaffe, 1976).

In contrast to the eggs of these species, mammalian eggs respond to fertilisation with phasic repetitive rises in intracellular Ca^{2+} . This was first demonstrated by Cuthbertson, Whittingham and Cobold (1981), using mouse eggs injected with aequorin. These transient rises preceded an exponential rise in intracellular $[\text{Ca}^{2+}]$ from a resting level of 0.1 μM to values of 5 μM and above. In hamster eggs, the transient rises in $[\text{Ca}^{2+}]_i$ did not precede a large rise in Ca^{2+} of this sort. Using Ca^{2+} -sensitive electrodes, Igusa and Miyazaki (1986) showed that the increase in Ca^{2+} during the first three transients was 1-2 μM , decreasing to a peak value of 0.7-0.8 μM during subsequent responses (the basal $[\text{Ca}^{2+}]_i$ was between 0.1 and 0.3 μM).

The suggestion made for the mouse egg (Cuthbertson et al., 1981) and the medaka fish egg (Gilkey, Jaffe, Ridgway and Reynolds, 1978) that the Ca^{2+} rise starts at site of sperm-egg fusion, has been confirmed in the hamster (Miyazaki, Hashimoto, Yoshimoto, Kishimoto, Igusa and Hiramoto, 1986). In this later work, high temporal resolution was obtained by recording light emission from aequorin-loaded eggs, using an advanced photo-imaging system.

The rise in intracellular Ca^{2+} at fertilisation has several consequences. Once again, this has been studied most thoroughly in the sea urchin egg. In this preparation, a rise in intracellular Ca^{2+} leads to exocytosis of cortical granules (and consequent fertilisation membrane formation), the interconversion of nicotinamide nucleotides (NAD to NADP) and a rise in intracellular pH of 0.5 pH units. This rise in intracellular pH is thought to be produced by an electrically neutral Na^+ - H^+ exchange (Whitaker and Steinhardt, 1982). Another possibility, however, is the activation of a voltage-dependent H^+ current, similar to the one observed in the oocyte of the amphibian, *Ambystoma* (Baud and Barish, 1984).

Ca^{2+}_i and the fertilisation potential.

The rise in $[\text{Ca}^{2+}]_i$ at fertilisation is also responsible for the activation of some of the ionic conductances underlying the fertilisation potential. There are two lines of evidence which have led to this proposal for many species of egg. Firstly, components of the fertilisation potential can be abolished by intracellular application of the Ca^{2+} chelator EGTA. Secondly, ionic conductances, dependent on the $[\text{Ca}^{2+}]_i$ and with similar characteristics to those seen at fertilisation, have been investigated in the unfertilised egg. This latter method involves producing a rise in $[\text{Ca}^{2+}]_i$, either by injection of Ca^{2+} or application of a Ca^{2+} ionophore such as A23187.

As described earlier, the fertilisation potential of most marine species results, in part, from an influx of Na^+ in to the egg to produce a characteristic depolarisation of the membrane (Hagiwara and Jaffe, 1979). Strong evidence for the contribution of a Na^+ current activated by Ca^{2+} , in the fertilisation potential has been obtained in the egg of the annelid, *Cerebratulus lacteus*. Injection of Ca^{2+} into the unfertilised *Cerebratulus* egg produces a Ca^{2+} -activated Na^+ current (Jaffe, Kado and Kline, 1986). The amplitude of the fertilisation potential in this egg is reduced on reduction of $[\text{Na}^+]_o$, suggesting the involvement of a Na^+ current. When the $[\text{Ca}^{2+}]_i$ in fertilised eggs was reduced to 0.1 μM or lower (using either the Ca^{2+} buffer BAPTA or EGTA), the fertilisation potential also decreased (Kline, Jaffe and Kado, 1986), indicating a dependence of this Na^+ current on $[\text{Ca}^{2+}]_i$.

A Ca^{2+} -dependent Cl^- current is thought to be activated at fertilisation in anuran amphibian eggs. Several Cl^- currents mediated by Ca^{2+} have been reported in unfertilised frog (Cross, 1980) and toad (Barish ^{and Thompson}, 1983) eggs. Cross (1980) demonstrated that a depolarisation in frog eggs, similar to that observed at fertilisation, could be evoked by Ca^{2+} injection.

The Ca^{2+} -dependence of the K^+ current underlying the fertilisation potential in some mammalian species has been well established. Injection of Ca^{2+} into unfertilised hamster eggs (Igusa and Miyazaki, 1983) produces a single transient hyperpolarisation of the membrane that closely resembles the hyperpolarisations observed during fertilisation (Miyazaki and Igusa, 1981). In the mouse egg, it is likely that there are fewer K^+ channels, compared to the hamster egg; iontophoretic injection of Ca^{2+} into unfertilised mouse eggs produced no effect with 4 nA of current (Igusa et al, 1983a) while only 0.4 nA injection current produced a substantial increase in K^+ conductance in unfertilised hamster eggs (Igusa et al., 1983b). The reversal potential measurements and ionic dependencies of sperm- and Ca^{2+} injection-induced hyperpolarisations are compatible with activation of a K^+ current. Injection of EGTA into the egg abolished the hyperpolarisations due to sperm activation of the egg. The occurrence of the hyperpolarising responses requires the presence of external Ca^{2+} as well. That they are abolished after several

minutes in the absence of $[Ca^{2+}]_o$ (Igusa and Miyazaki, 1983) suggests that they depend on Ca^{2+} influx.

It is obvious that any manipulations of these cells, leading to an alteration in $[Ca^{2+}]_i$, might be used as tools to investigate these Ca^{2+} -activated currents.

1.2 Modulation of $[Ca^{2+}]_i$.

The $[Ca^{2+}]_i$ of most cells is affected by a variety of systems;

- influx through Ca^{2+} channels.
- uptake of Ca^{2+} into, and release of Ca^{2+} from, intracellular stores such as endoplasmic reticulum or mitochondria.
- efflux via Ca^{2+} extrusion systems, such as active Ca^{2+} pumps and Na^+ - Ca^{2+} exchangers.

Unlike the first two systems, Ca^{2+} extrusion systems have not been implicated in the activation currents at fertilisation; Ca^{2+} influx and release of Ca^{2+} from intracellular stores are accepted as contributors to the characteristics of activated eggs.

Early experiments of Ca^{2+} transport indicated that Na^+ or ATP may be directly involved in regulation of $[Ca^{2+}]_i$. The first proposal of the hypothesis that the energy stored in the electrochemical gradient for Na^+ might lead to Ca^{2+} extrusion, via a Na^+ - Ca^{2+} countertransport system, was made by Reuter and Seitz (1968) working on cardiac muscle. Since then, this explanation for the involvement of Na^+ associated with Ca^{2+} extrusion has been applied in a variety of tissues (Villereal, 1986). A different system is reported for red blood cells (Schatzman and Burgin, 1978) and sarcoplasmic reticulum (Kurzmaek, Verjovski-Almeida and Inesi, 1979) where the transport of Ca^{2+} is not apparently coupled to the electrochemical gradient of another ion, but is instead dependent on ATP for energy. In general, these results were obtained by measuring ion fluxes using radioactively-labelled tracer ions.

A valuable source of information on Ca^{2+} extrusion in excitable cells comes from internal dialysis experiments on squid axons (DiPolo and Beauge, 1983). In this preparation, both a Na^+ - Ca^{2+} exchange and an active Ca^{2+} pump which regulate intracellular Ca^{2+} with different degrees of efficiency are present, both systems working in parallel. The Ca^{2+} pump is thought to be responsible for maintaining the resting $[Ca^{2+}]_i$, while the Na^+ - Ca^{2+} exchange system deals with large increases in intracellular $[Ca^{2+}]_i$. Parallel independent Ca^{2+} extrusion systems have also been

proposed for cardiac muscle (Caroni and Carafoli, 1980), cultured neuroblastoma cells (Kurzinger, Stadtkus and Hamprecht, 1980) and mammalian CNS nerves (Schellemborg and Swanson, 1982).

In addition to the different dependencies of the Na^+ - Ca^{2+} exchange and the Ca^{2+} pump, on Na^+ and ATP respectively, these two systems may be distinguished by different inhibitors. A good example of this comes from results obtained using dialysed squid axons. Raising pH_o above 8.0 produces a decrease in the efflux of Ca^{2+} via the active Ca^{2+} pump, with little effect on the efflux of Ca^{2+} via the Na^+ - Ca^{2+} exchange (DiPolo and Beauge, 1982). This inhibition of efflux is markedly enhanced by the presence of Ca^{2+} in the bathing solution. In contrast, raising from pHi 6.8 to 8.8 markedly facilitated the efflux due to the Na^+ - Ca^{2+} exchange (producing a four-fold increase), while no effect was observed on the uncoupled component of Ca^{2+} extrusion.

The removal of extracellular Na^+ in order to inhibit the Na^+ - Ca^{2+} exchange system of cardiac cells is now an established procedure. Reuter and Seitz (1968) first demonstrated that bathing cardiac cells in Na^+ -free solution produced a reversible contracture and attributed this to a rise in Ca^{2+} due to inhibition of Ca^{2+} efflux.

There are several groups of chemicals that can act as blockers of the ATP-dependent Ca^{2+} pump. Among these are some inorganic ions, such as lanthanum and vanadate, calmodulin antagonists, such as phenothiazines, and plant flavones such as quercetin. Unfortunately, none of these are specific blockers of the active Ca^{2+} pump on cell plasma membranes; most inhibitors affect other ATP-dependent pumps, such as the Na^+ - K^+ ATPase or the Ca^{2+} ATPase of endoplasmic reticulum.

Lanthanum has been used extensively as an inhibitor of the Ca^{2+} pump in studies of Ca^{2+} efflux from red blood cells (Sarkadi, Szasz, Gerloczy and Gardos, 1977), adipocytes (Pershadsingh and McDonald, 1980) and synaptic vesicles (Sorensen and Mahler, 1981). It is effective when applied externally or internally. It should be noted that La^{3+} is also known to block the Na^+ - Ca^{2+} exchange in cardiac cells (Kimura, Noma and Irisawa, 1986). Vanadate has also been used as a blocker of the active Ca^{2+} pump, but is only effective at the internal membrane surface (DiPolo and Beauge, 1981).

Inhibition of the Ca^{2+} pump by quercetin has been demonstrated in red blood cells (Wulrich and Schatzmann, 1980) and fibroblastic L cells (Okada, Tsuchia and Yada, 1982). However, quercetin is also known to inhibit other ATPases, such as the Na^+ - K^+ ATPase of the electric organ of electric eels (Kuriki and Rakov, 1976). *and Gomperts, 1977).*

Application of these inhibitors of Ca^{2+} extrusion may be used to induce a rise in $[\text{Ca}^{2+}]_i$; the activation of channels by intracellular Ca^{2+} , such as the Ca^{2+} -activated K^+ channel of the hamster egg, may then be investigated.

1.3 Ca^{2+} -activated K^+ channels.

Early studies.

The first demonstration of a Ca^{2+} -activated K^+ conductance using electrophysiological techniques was by Meech and Strumwasser (1970). They pressure-injected Ca^{2+} into *Aplysia* neurones using a micropipette and recorded the resulting changes in membrane potential and input resistance, using intracellular recording techniques. They concluded that the injected Ca^{2+} caused a rise in membrane conductance for K^+ .

Similarly, an increase in membrane conductance, associated with a change in membrane potential, was obtained in mammalian spinal motoneurones, using iontophoretic injection of Ca^{2+} (Krjnevich and Lisiewicz, 1972). The ionic distributions in this preparation suggested that the response was due to an increase in either K^+ or Cl^- conductance. The difference in reversal potential of the response to Ca^{2+} injection, and that of a chloride-dependent inhibitory post synaptic potential (i.p.s.p), led these workers to attribute the Ca^{2+} -evoked response to an increase in K^+ conductance. It was Meech (1974) who demonstrated the dependence of the reversal potential of the Ca^{2+} -evoked response in *Helix* neurones on K^+ .

Previous reports of the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) required to evoke a response had been in the millimolar range; Meech (1974) showed that pressure-injection of a calcium-EGTA buffer containing 0.7 μM free Ca^{2+} was sufficient. Additionally, using repetitive depolarising steps under voltage-clamp, to simulate the effect of action potentials, he showed that a sufficient Ca^{2+} influx could be induced to activate a K^+ conductance. This effect was potentiated by application of metabolic inhibitors such as 2,4-dinitrophenol. These agents had been known for some time to produce a K^+ efflux in red blood cells (Passow, 1963), and are thought to act by reducing Ca^{2+} buffering capacity.

The combination of using metabolic inhibitors (and other agents capable of disrupting Ca^{2+} buffering) and Ca^{2+} injection techniques has great potential for elucidating the relation between cell control of $[\text{Ca}^{2+}]_i$ and activation of these K^+ channels.

Another useful combination of techniques, applied by Gorman and Thomas (1979) to *Aplysia* neurones, was to monitor ionic currents evoked by Ca^{2+} injection with a voltage-clamp system, and simultaneously monitor $[\text{Ca}^{2+}]_i$ with a Ca^{2+} -dependent photosensitive dye. Using the

metallochromic calcium-sensitive dye, Arsenazo III, they observed a strong correlation between the timecourse of the change in $[Ca^{2+}]_i$ and the timecourse of the Ca^{2+} -activated K^+ current. In addition, a voltage-sensitivity of the Ca^{2+} -activated K^+ conductance was found at certain $[Ca^{2+}]_i$ at 1.6 μM , the K^+ current increased e-fold for every 25 mV change in membrane potential.

Although much information was obtained about the Ca^{2+} -activated K^+ current using conventional intracellular recording and voltage-clamp techniques, development of the patch-clamp recording technique provided a means for investigating the Ca^{2+} -activated K^+ current at the single channel level.

Single channel studies of the Ca^{2+} -activated K^+ current.

The first recording of single Ca^{2+} -activated K^+ channels was obtained from bovine adrenal chromaffin cell membranes (Marty, 1981). It was demonstrated that these channels open in response to an increase in $[Ca^{2+}]_i$ and/or membrane depolarisation.

Channels with similar properties to the chromaffin channel have subsequently been found in many different cells, including skeletal muscle cells (Barrett, Magleby and Pallotta, 1982), neurones (Smart, 1987), macrophages (Gallin, 1984) and endocrine (Wong, Lecar and Adler, 1982) and exocrine cells (Maruyama and Petersen, 1983). ^{Gallacher} Ca^{2+} -activated K^+ channels from muscle cells have also been reconstituted into planar lipid bilayers (Vergarra and Latorre, 1983).

Other channels have been reported that are permeable to K^+ and activated by Ca^{2+} , that are not selective for this ion (see Petersen and Maruyama, 1984; Partridge and Swandulla, 1988 for reviews); these non-selective cation channels are not included in this review.

From single channel studies, Ca^{2+} -activated K^+ channels may be divided into two general groups on the basis of single channel conductance; firstly, a large conductance K^+ channel (100-250 pS) and secondly a small conductance K^+ channel (10-50 pS). These values were obtained with symmetrical solutions containing 100-150 mM K^+ on either side of the membrane patch. Under these conditions, the potential of the patch, without applied voltage, is 0 mV.

The typical current-voltage relationship of the large conductance channels is linear over a wide range of patch membrane potential (-60 to +40 mV). Any further hyperpolarisation or depolarisation has been shown to result in a deviation in the slope conductance from linearity, i.e. rectification. The open probability of large conductance Ca^{2+} -activated K^+ channels is increased by membrane depolarisation. Thus the activity of these channels is under the dual control of membrane potential and $[Ca^{2+}]_i$.

Although small-conductance Ca^{2+} -activated K^+ channels are also Ca^{2+} activated, the channel open probability has been shown to be independent of membrane potential.

Control of Ca^{2+} -activated K^+ channels.

The activity of large conductance Ca^{2+} -activated K^+ channels is controlled by two main factors, membrane potential and the intracellular free Ca^{2+} concentration. Altering the external $[\text{Ca}^{2+}]$ does not affect the channel activity (Barrett, Et al., 1982).

(1) Voltage dependency

Prior to the development of the patch-clamp recording technique, the question arose as to whether the observed voltage-dependency of the Ca^{2+} -activated K^+ conductance was a property of these channels, or merely a consequence of Ca^{2+} influx through voltage-gated Ca^{2+} channels. In answer to this, it was shown that depolarisation could evoke Ca^{2+} -activated K^+ channel opening in the absence of internal Ca^{2+} (Maruyama, Gallacher and Petersen, 1983) using inside-out patches of salivary gland acinar cells. Thus voltage dependency may, in some membranes, be the more important activator of the potassium channel.

In general, the channel open probability (P_o) of large conductance Ca^{2+} -activated K^+ channels increases in a sigmoidal fashion with membrane depolarisation. This is the case in reconstituted channels from rat skeletal muscle (Moczydlowski and Latorre, 1983), clonal anterior pituitary cells (Wong, Lecar and Adler, 1982) and cultured rat muscle (Barrett, Et al., 1982). This sigmoid curve of the relation between P_o and membrane potential is described by equation 1,

However, there are some exceptions to this; instead of a sigmoid curve, the large conductance Ca^{2+} -activated K^+ channel from cultured monkey renal epithelium has a bell-shaped relationship when plotted against percent open time (Kolb, Brown and Murer, 1987). This means that instead of attaining a steady value for the permanently open channel state at depolarisations of +40 mV or more (Moczydlowski and Latorre, 1983), the channel open time declines. This occurs in the presence of the minimum Ca^{2+} concentrations required to activate the channel; the decrease in P_o at positive potentials may therefore not be due to Ca^{2+} -induced blockade of the Ca^{2+} -activated K^+ channel. As the number of channels active in the patch was unknown, further interpretation of this phenomenon is difficult.

Interestingly in rat skeletal muscle, it was reported that the Ca^{2+} -sensitivity of the K^+ channel could be irreversibly removed by N-bromoacetamide (N-BA) leaving just a voltage-sensitive channel (Pallotta, 1985).

Cornejo, Guggino and Guggino (1987) found a similar effect of N-BA on Ca^{2+} -activated K^+ channels from cultured medullary thick ascending limb (MTAL) cells. However, they demonstrated that the effect of N-BA was to decrease the Ca^{2+} -sensitivity of the K^+ channel, rather than abolish it all together; raising the $[\text{Ca}^{2+}]_i$ concentrations from 1 μM to 25 mM could evoke channel opening.

The small conductance Ca^{2+} -activated K^+ channel has been shown to be nearly voltage insensitive in the voltage range -100 to +100mV (Sauve et al, 1986). However, the open probability has been shown to rise at hyperpolarising voltages (Grygorczyk & Schwarz, 1983, Sauve et al, 1986), in contrast to the open probability of the large conductance channels. In addition, there is an example of a low conductance Ca^{2+} -activated K^+ channel in *Necturus oxyntic* cells which is activated by depolarisation (Ueda, Loo and Sachs, 1987).

It should be noted that there are examples of voltage insensitive Ca^{2+} -activated K^+ channels in the literature (Colquhoun et al, 1981) but these channels were found to be equally permeable to several cations (non-selective cation channels) and are therefore different to the highly specific K^+ channels found here.

(2) Internal calcium concentration

The dependence of large conductance channels on the intracellular Ca^{2+} concentration was first demonstrated by Marty (1981) in chromaffin cells. He showed that, using inside-out patches, application of Ca^{2+} (0.001 to 0.01 μM) to the cytoplasmic side caused channel opening. Marty also demonstrated that this calcium dependency was only at the intracellular side. A further discovery was that when the internal concentration of calcium was raised above 1mM the single channel conductance decreased, the mean number of open channels increased and the voltage dependence of opening was lost.

The sensitivity to the internal concentration of calcium seems to vary considerably from one preparation to another. The early results from bovine chromaffin cell membranes indicated that depolarisation induces full channel opening at a $[\text{Ca}^{2+}]_i$ concentration of 0.01 μM (Marty, 1981). However, in nominally Ca^{2+} -free solutions, a marked activation of Ca^{2+} -activated K^+ channels occurred on depolarisation of inside-out patches of membrane from mammalian salivary glands (Maruyama, Gallacher and Petersen, 1983); this activity was dramatically enhanced when $[\text{Ca}^{2+}]_i$ was raised to 0.1 μM .

In contrast to these results, channel recordings from cultured rat muscle cells show a much lower sensitivity to Ca^{2+} ; depolarisation from -50mV to +50mV did not evoke channel opening at 0.01 μM Ca^{2+} , had little effect at 0.1 μM and required internal concentrations of 1.0 μM Ca^{2+} for channel activation (Barrett, Et al., 1982). Studies on human macrophages (Gallin, 1984), have

shown that the concentration of calcium (between 0.1 and 1 μM) necessary for activation is similar to that for cultured rat muscle. A study of renal epithelia (Kolb et al, 1986) reveals that for a given membrane potential, a low internal concentration of calcium ($<0.1 \mu\text{M}$) produces a greater open probability than that found in muscle cells or neurones (Barrett et al 1982, Moczydlowsky & Latorre, 1983). A possible explanation for this may be a higher binding affinity for Ca^{2+} of the Ca^{2+} -activated K^+ channel in the renal epithelial cells.

Studies of rat sympathetic neurons have revealed a Ca^{2+} -activated K^+ channel which was virtually inactive when the $[\text{Ca}^{2+}]_i$ was below 0.1 μM . However when the $[\text{Ca}^{2+}]_i$ was raised above 0.1 μM the channel could be activated by depolarising the cell membrane.

High levels of $[\text{Ca}^{2+}]_i$ will actually reduce P_o at very positive potentials in pancreatic islet cells (Findlay, Dunne and Petersen, 1985) and pancreatic acinar cells (Maruyama, Petersen, Flanagan and Pearson, 1983). In the latter preparation, large depolarisations with a $[\text{Ca}^{2+}]_i$ of 0.1 μM and above sometimes led to a state of prolonged channel closure. A similar blockade of this channel was produced with Ba^{2+} , where the decrease in P_o was due to a prolongation of closed times (Iwatsuki and Petersen, 1985).

To summarise, in secretory cells such as chromaffin (Marty, 1981), salivary (Maruyama, et al, 1983) and pancreatic (Maruyama et al, 1983b) acinar cells, it appears that the K^+ channel is under the control of both the membrane potential and the internal concentration of calcium. Under physiological conditions, an increase in the $[\text{Ca}^{2+}]_i$ will increase the open state probability, which will hyperpolarise the membrane, and in turn, reduce the open probability.

Small conductance K^+ channels also show some variation in their sensitivity to the internal concentration of calcium. In HeLa cancer cells (Sauve, Simoneau, Monette and Roy, 1986) channel activity could be initiated at concentrations ranging from 0.1 to 1 μM , whereas in red blood cells, the effect of the internal concentration of calcium was in the range 1 to 10 μM (Grygorczyk, Schwarz and Passow, 1984).

Other modulators of Ca^{2+} -activated K^+ channels.

The activity of these channels may be modulated by endogenous factors other than Ca^{2+} and membrane potential.

The possible involvement of Mg^{2+} in modulating the activity of Ca^{2+} -activated K^+ channels from rat skeletal muscle reconstituted into planar phospholipid bilayers was presented by Golowasch, Kirkwood and Miller (1986). The effective concentration range of Mg^{2+} in these channels was 5-10 mM, where it not only potentiates Ca^{2+} activation of the channels, but controls

the cooperativity of Ca^{2+} activation. This effect is apparent after application of Mg^{2+} to the intracellular surface of the membrane. These workers suggest that Mg^{2+} reveals Ca^{2+} binding sites on the protein that are masked in the absence of Mg^{2+} .

However, estimates of $[\text{Mg}^{2+}]_i$ in vivo of 0.4-3 mM (Corkey, Duszynski, Rich, Matschinsky and Williamson, 1986), lead to the question of the physiological significance of such high concentrations Mg^{2+} as 10 mM. To answer this, Golowalsch et al proposed that the channel reconstituted into the bilayers (composed of neutral lipids) was less sensitive than the channel in its natural environment (surrounded by negatively-charged lipids), due to electrostatic surface potential effects on the channel. This proposition is probably justified; Squire and Petersen (1987) recently demonstrated modulation of Ca^{2+} -activated K^+ channels by Mg^{2+} in more physiological concentration ranges (1 μM to 1 mM). These results were obtained from inside-out patches of membranes excised from salivary acinar cells; a dose-dependent K^+ channel activation by Mg^{2+} was obtained at a constant Ca^{2+} concentration of 0.01 μM . It may be that fluctuations of intracellular Mg^{2+} in intact cells influence channel activity.

The conductance of Ca^{2+} -activated K^+ channels has been reported to be very temperature-sensitive in several preparations; the channel conductance increases with temperature. For example in cultured rat muscle, the conductance is 100 pS at 1 °C and is 300 pS at 37 °C (Barrett, et al., 1982). Similarly, reducing the temperature over the range 0-47 °C decreases the single channel conductance in erythrocytes (Grygorczyk, 1987). However, in this preparation, the situation is complicated; the reduction in temperature also alters channel gating, resulting in an increase in P_o .

Protons have been suggested to inhibit Ca^{2+} -activated K^+ conductance in pancreatic β -cells (Cook, Ikeuchi and Fujimoto, 1984). The channel open probability of a 240 pS channel, for a given voltage and fixed $[\text{Ca}^{2+}]_i$, decreased as the intracellular surface of the membrane was acidified. A similar effect was seen with a 200 pS channel from the epithelium of choroid plexus ventricular membrane (Christensen and Zeuthen, 1987); lowering the intracellular pH from 7.4 to 6.4 reduced P_o . This reduction was mainly due to a large increase in channel closed time (a factor of ten for this pH change). A change in pH from 7.4 to 8.4 did not influence P_o significantly. In the cells these channels are obtained from, pH may be a regulatory factor.

In contrast to this, no pH dependence was found for Ca^{2+} -activated K^+ channels from an insulin-secreting cell line (Light, Van Eenenaan, Sorenson and Levitt, 1987) in the range 6.1 to 8.0. In this cell line, ATP-sensitive K^+ channels are reversibly inhibited by H^+ .

In HeLa cells, a potassium channel activated by high external $[\text{Ca}^{2+}]$ and alkaline pH has been reported (Simoneau, Sauve and Roy, 1984) in an abstract, but no further information is available.

The involvement of protein phosphorylation in modulating Ca^{2+} -activated K^+ channels was indicated in voltage-clamp studies on internally perfused *Helix aspera* neurones (de Peyer, Cachelin, Levitan and Reuter, 1982). Single-channel recordings indicate that the channel concerned is a low conductance Ca^{2+} -activated K^+ channel (40-60 pS) and responds to the catalytic sub-unit (CS) of cAMP-dependent protein kinase (Ewald, Williams and Levitan, 1985). Bath application of 0.4 μM or less of the CS to an excised inside-out patch produced long lasting enhancement of channel activity. This was due to an increase in both channel open probability and the number of channels. Information on the site of phosphorylation comes from studies with reconstituted channels (Ewald et al, 1985). The site of phosphorylation is either on the channel protein itself or on some regulatory protein that is tightly bound to the channel, as it seems likely that only tightly bound regulatory components would remain after channel reconstitution.

Subconductance States.

The existence of a subconductance state, smaller than the main open state, was first reported by Hamill and Sakmann (1981) for single acetylcholine receptor channels in embryonic muscle cells. Since then, subconductance states have been observed in inward rectifier potassium channels (Sakmann and Trube, 1984), non-selective cation channels (Rae and Laevis, 1984) and anion channels (Hamill, Bormann and Sakmann, 1983).

Similarly, Ca^{2+} -activated K^+ channel subconductances were also seen in cultured rat muscle (Barrett, et al., 1982). This subconductance state had a current amplitude about 40 % of the normal state. Openings of this amplitude comprised less than 0.1 % of the total channel open time.

The criteria for recognizing substates was applied to these channels (Fox, 1987); direct transitions from one conductance state to another were observed, the substate was only observed in the presence of channel main-state activity and no evidence of superimposition of channels was seen. There are some possible pitfalls in interpreting channel records in terms of subconductance states; for example, it is possible to confuse an altered kinetic state with a subconductance state due to bandwidth limitations in the recording apparatus (Fox, 1987).

Kinetics of activation of the Ca^{2+} -activated K^+ channel.

Activation of the Ca^{2+} -activated K^+ channel has been studied in great detail by direct observation of the opening and closing of single channels. This information has been used to describe models for the mechanism of channel activation (Barrett, et al., 1982; Methfessel and Boheim, 1982; Moczydlowski and Latorre, 1983). The channel's conducting, or open, conformation is favoured as the concentration of Ca^{2+} at the cytoplasmic side of the membrane is increased. This activation process is cooperative (Barrett, et al., 1982); a sigmoid curve is obtained when the

channel open probability (P_o) is plotted against $[Ca^{2+}]$ at a given voltage. Additionally, at any given voltage, V , the open-state probability is described by a sigmoid curve of the form,

$$P_o = (1 + \exp(\frac{-zF(V-V_c)}{RT}))^{-1}$$

equ.(1)

where V_c is the voltage at which half the channels are open, z is the effective charge on the gating particle, F is the Faraday constant, R is the Gas constant and T is the absolute temperature (Wong, Lecar and Adler, 1982). The effect of increasing the $[Ca^{2+}]_i$ is to produce a parallel shift of the curve described by equ.1 along the voltage axis to more negative potentials.

From these two curves, estimates for the number of calcium ions involved in activating the channel may be made. For example, Wong et al. (1982) found a tenfold increase in free $[Ca^{2+}]_i$ shifts the curve described by equ. 1 by 65 mV in Ca^{2+} -activated K^+ channels of cloned anterior pituitary cells. They explain this by assuming that the Ca^{2+} binding step is voltage sensitive and highly cooperative (a number of Ca^{2+} are required to open the channel; 3 in this preparation).

A similar conclusion was reached by Moczydlowski and Latorre (1983), working with rat muscle cell channels reconstituted in to phospholipid bilayers. After plotting P_o against $[Ca^{2+}]_i$, they obtained a Hill coefficient of 1.2, which led them to suggest that at least two Ca^{2+} ions were required to open a K^+ channel. They proposed that the binding of Ca^{2+} to the channel was voltage dependent.

In contrast, Methfessel and Boheim (1982) claim that the voltage dependence is in the open-closed conformational changes, and that although at least two of the Ca^{2+} ions are required, the binding of these is not voltage dependent. They propose a two-step gating mechanism involving activation by Ca^{2+} and blockade by a Ca^{2+} -sensitive gate.

More recent kinetic analysis indicates that the earlier suggestions for the number of Ca^{2+} binding sites of the Ca^{2+} -activated K^+ channel were underestimates. McManus and Magleby (1985) performed further kinetic analysis on cultured rat muscle channels; in the absence of Mg^{2+} , a Hill plot of the % time open against $[Ca^{2+}]_i$ had a slope of 3.6, suggesting at least four Ca^{2+} ions binding for maximum activation. While investigating the effects of Mg^{2+} on the kinetics of reconstituted Ca^{2+} -activated K^+ channels, Golowasch et al (1986) found Hill coefficients (for P_o against $[Ca^{2+}]_i$) as high as 5.8, suggesting at least six Ca^{2+} binding sites are involved in the activation of this channel.

Models describing the number of open and closed states of the Ca^{2+} activated K^+ channel have been developed by fitting exponentials to the distribution of open and closed states. Thus the earlier work of Barrett et al (1982) proposed a model with two different open channel states of apparently normal conductance. By 1986, Golowasch et al. had proposed a model with at least three open and six shut states, based on the distribution of open and shut times.

The discrepancy between these proposals for kinetic models may be due to different materials; however, it may also highlight one of the problems associated with the analysis of single channel recordings, namely frequency resolution. Ideally, the signal should be filtered at a frequency that removes unwanted noise, while leaving genuine channel events unaffected. Even though the Ca^{2+} -activated K^+ channel has a large conductance and a resulting large signal-to-noise ratio compared to other channels, it is still difficult to distinguish fast channel events from high frequency noise.

Function of the Ca^{2+} -activated K^+ channel.

The apparent function of the calcium dependent potassium varies with the preparation, depending mainly on whether the channel is activated at the normal internal concentration of calcium.

Neurones and muscle cells.

Ca^{2+} -activated K^+ conductance has been implicated in the regulation of repetitive activity in a wide variety of vertebrate and invertebrate neurones (Meech, 1980) and in cultured rat muscle (Barrett, Barrett and Dribin, 1981). The large conductance and high calcium and voltage sensitivity of the channel would support this hypothesis. The Ca^{2+} influx which accompanies an action potential, would activate these channels, resulting in a hyperpolarising potassium current, which would tend to resist further depolarisation. The magnitude of the hyperpolarisation following the action potential would be graded, too.

Although this is a tempting hypothesis, the presence of a Ca^{2+} -activated K^+ channel in a cell does not necessarily indicate a dramatic involvement in regulating the membrane potential. It was previously thought that the Ca^{2+} -activated K^+ channel was responsible for the interburst hyperpolarisation observed in certain *Aplysia* neurones (Gorman and Thomas, 1978). However, this interburst hyperpolarisation is insensitive to changes in extracellular K^+ or TEA, unlike the Ca^{2+} -activated K^+ current induced by Ca^{2+} injection in these neurones (Adams and Levitan, 1985). An alternative proposal is that a Ca^{2+} -dependent inactivation of a resting Ca^{2+} influx occurs between bursts.

If the Ca^{2+} buffering capacity of excitable cells is influenced by the metabolic state of the cell, then the Ca^{2+} -activated K^+ channel may form a link between metabolism and membrane excitability (Meech, 1978). Under this scheme, As the energy of the cell is depleted, the Ca^{2+} buffering ability of the cell is reduced and the K^+ channel is activated. This hyperpolarises the cell, reducing activity.

Work on rat sympathetic ganglion neurones has revealed rapid relaxation kinetics which may suggest a role in the repolarisation of the action potential (Smart, 1987) especially considering the voltage-sensitivity of these channels. These Ca^{2+} -activated K^+ channels are activated at the resting potential of the neurones (-45 to -65 mV) which is unusual in comparison with most other cells (Barrett, et al. 1982). This would indicate a possible contribution of this channel to the resting potential.

Secretory cells

These cells may be conveniently divided up into two groups; those with the capacity to generate action potentials, and those without this ability. Broadly, most endocrine cells generate action potentials, whereas exocrine cells do not.

The K^+ channel in endocrine cells is thought to act as a link between the $[\text{Ca}^{2+}]_i$ and the membrane potential. For example, in the chromaffin cell and the pancreatic β -cell, Ca^{2+} required for secretion enters through specific voltage-gated channels (Reuter, 1983) which are opened by depolarisation and closed by hyperpolarisation. The resulting rise in $[\text{Ca}^{2+}]_i$ due to this influx will activate Ca^{2+} -activated K^+ channels, leading to a membrane hyperpolarisation. This in turn limits Ca^{2+} influx, preventing Ca^{2+} loading of the cell and excessive secretion.

It has been proposed that glucose produces a decrease in the Ca^{2+} sensitivity of this channel and enhances secretion of insulin by prolonging the depolarisation that leads to Ca^{2+} influx (Atwater, Ribalet and Rojas, 1978). However, investigations at the single channel level have shown that a K^+ channel inhibited by ATP is present in these cells (Rorsman and Trube, 1985). This channel may be more important in pancreatic β -cells in controlling membrane potential; glucose metabolised by the cell would produce an increase in the $[\text{ATP}]_i$. This would inhibit K^+ channels, depolarising the cell.

In exocrine cells with no capacity to generate action potentials, the Ca^{2+} -activated K^+ channels must have a different function to that proposed for endocrine cells. In general, the secretion from these cells is accompanied by a marked increase in extracellular K^+ (Young, 1979; Petersen, 1980). This secretion is under strict nervous or hormonal control; for example in salivary gland acinar

cells, nerve stimulation or direct stimulation with acetylcholine, noradrenaline or substance P produces dramatic loss of K^+ from the cell.

This loss of K^+ and the secretion from the acini is dependent on the presence of Ca^{2+} . The discovery of Ca^{2+} -activated K^+ channels in these cells (Maruyama, et al, 1983a; Maruyama and Petersen, 1984) provides a possible explanation for the K^+ loss from these cells on stimulation. The stimulus-evoked change in the transmembrane K^+ gradient may provide the means of influencing transport systems, allowing control over secretion of fluids. This has led to the proposal of a regulation of transepithelial fluid by control of K^+ channels (Suzuki and Petersen, 1985).

Studies of cultured renal epithelium (Kolb, et al, 1986) have revealed that as with other membranes, depolarisation and increases in $[Ca^{2+}]_i$ activate a K^+ conductance which leads to a hyperpolarisation. This may be a means of maintaining the apical membrane potential and hence the driving force for the sodium-solute co-transport across this membrane. There could also be an effect on the calcium-sodium exchanger.

The Ca^{2+} -activated K^+ channel of the oxyntic cell (Ueda, Loo and Sachs, 1987) may account for the hyperpolarisation and conductance increase of the basolateral membrane during acid secretion. This acid secretion is dependent on an exchange between cytosolic H^+ and luminal K^+ , and is mediated by a rise in intracellular Ca^{2+} . Thus activation of this channel may provide control over the acid secretion via an effect on the K^+ concentration gradient across the basolateral membrane.

Other cells

Macrophages are well known for their secretory, phagocytotic and motile properties. Changes in intracellular calcium levels have been implicated in some of these functions. Stimulation of macrophages by the calcium ionophore A23187 (Gallin & Gallin, 1977) has been shown to produce a membrane hyperpolarisation. Therefore the Ca^{2+} -activated K^+ conductance, once triggered, might modulate other voltage-dependent conductances by producing such a hyperpolarisation. Alternatively, or in addition, the Ca^{2+} -activated efflux of K^+ may influence surrounding cells.

The function of the Ca^{2+} -activated K^+ channel has been investigated in the ciliated protozoan *Paramecium*, using behavioural mutants. *Paramecium* displays certain behavioural responses to various stimuli; chemicals, heat, light, touch leads to the generation of a transient membrane depolarisation (Kung and Saimi, 1982). This activates a graded Ca^{2+} action potential; the influx of Ca^{2+} causes the cilia propelling the cell to beat in the opposite direction. Eventually, the cell repolarises and begins to swim forward again.

This repolarisation is thought to result, at least in part, from the activation of a Ca^{2+} -activated K^+ current; one class of mutants has a decreased Ca^{2+} -activated K^+ current and continues to swim backwards for far longer than the wild type *Paramecium* (Saimi, Hinrichsen, Forte and Kung, 1983). Another mutant has a larger Ca^{2+} -activated K^+ current (due to increased sensitivity to Ca^{2+} and voltage compared to the wild type and has a reduced behavioural response (Hennessy and Kung, 1987). This is possibly due to an increased rate of repolarisation of the membrane, decreasing Ca^{2+} influx.

Channel selectivity.

The selectivity of a channel for various cations can be measured by a shift in the reversal potential (Hille, 1970) when a test ion, X, is substituted for internal potassium. This shift in reversal potential is used to determine the relative permeability of X to K^+ under the conditions of zero current. The relationship between the permeability ratio and the shift in the reversal potential is obtained from a derivation of the Goldman-Hodgkin-Katz (G-H-K) equation (Hille, 1971, 1973). For the condition of X_i being replaced by K^+ the shift in the reversal potential is

$$V_X - V_K = \frac{RT}{F} \ln \left(\frac{P_K K_o}{K_i} \right) - \frac{RT}{F} \ln \left(\frac{K_o}{K_i} \right) \quad \text{equ (2).}$$

where V_X and V_K are the reversal potentials with X and K at the inner membrane surfaces respectively; P_X and P_K are the permeabilities of the channel to X and K respectively; K_o , K_i and X_i are the activities of the individual ions $F/RT = 0.0394 \text{ mV}^{-1}$ at 22°C .

The permeability ratio of X to K is then obtained by rearrangement:

$$\frac{P_X}{P_K} = \frac{K_i}{X_i} \exp \left(\frac{(V_K - V_X)F}{RT} \right) \quad \text{equ (3).}$$

When it is difficult to control the ion concentration at the inner surface membrane, an alternative method may be used; the reversal potential is calculated for each ion in turn. The difference in reversal potential between the two ions is given by

$$E_R = E_{R,B} - E_{R,A} = \frac{RT}{zF} \ln \left(\frac{P_B[B]_o}{P_A[A]_o} \right) \quad \text{equ (4).}$$

Thus the permeability ratio P_B/P_A is easily obtained. Using these methods the selectivity sequence of large conductance Ca^{2+} -activated K^+ channels in cultured rat muscle was shown to be:



with permeability ratios of 1.2, 1.0, 0.67, 0.11 (Blatz and Magleby, 1984). Monovalent cations Na^+ , Li^+ and Cs^+ were not measurably permeable, with permeabilities less than 5 % that of K^+ . The currents of the various ions were typically less than expected on the basis of permeability ratios, suggesting that the movement of an ion through the channel is not independent of other ions present. Similar results have also been obtained under voltage-clamp in *Aplysia* neurons (Gorman and Thomas, 1981). In the large conductance Ca^{2+} -activated K^+ channels of other tissues a high selectivity for potassium over sodium has been demonstrated.

Another measure of selectivity is the fluctuation in single channel conductance in channels reconstituted in planar lipid bilayers, which has been shown to depend on the permeant ion. A similar selectivity sequence ($Tl^+ > K^+ > NH_4^+$) was observed in reconstituted channels from rat skeletal muscle (Latorre, Vergarra and Moczydlowski, 1983). This cation selectivity sequence has also been reported for K^+ channels of squid axons and the inward rectifier K^+ channel of starfish eggs (Hagiwara and Takahashi, 1974).

The paradox of this high selectivity combined with high conductance has led Latorre and Miller (1983) to suggest that the large conductance Ca^{2+} -activated K^+ channel have large access mouths that lead to a narrow constriction of short length, with a further constriction at the point of the selectivity filter.

When comparing the two methods of measuring selectivity some inconsistencies occasionally appear; the results from the conductance fluctuation method and the reversal potential method are similar at low ionic concentrations. However, comparing the selectivity sequence at high ionic concentrations leads to a discrepancy; the conductance of the channel is blocked by these ions, changing the selectivity ratio.

In contrast to the Ca^{2+} -activated K^+ channels in skeletal muscle, the Ca^{2+} -activated channels from acinar cells have a very low Rb^+ conductance (Yellen, 1984; Gallacher, Maruyama and Petersen, 1984); with extracellular Rb^+ and intracellular K^+ , current will flow from the inside to the outside, but no inward Rb^+ current can be measured. With the Rb^+ and K^+ gradients reversed, an inward current but no outward current could be recorded.

The small conductance channels appear to have a similar selectivity for K^+ over Na^+ in molluscan neurones (Lux, Neher and Marty, 1981) and also in HeLa cancer cells where the P_{Na}/P_K ratio was found to be 0.03, which is consistent with a high potassium selectivity.

As the conductance for these Ca^{2+} -activated K^+ channels is smaller than that of the large conductance channels, the structure of the channel could be different; however, the similarity in the selectivity suggests a similar selectivity filter (Hille, 1984).

Activation of the Ca^{2+} -activated K^+ channel by ions other than Ca^{2+} .

Activation of the Ca^{2+} -activated K^+ channel of *Aplysia* neurones by Sr^{2+} and Ba^{2+} , but not Mg^{2+} , was reported by Meech (1974). However, a later study of by Meech and Thomas (1980) reexamined this effect of Ba^{2+} injection, and came to the conclusion that the Ca^{2+} -sensitive receptor of the K^+ channel was not sensitive to Ba^{2+} ; the hyperpolarising response sometimes produced by Ba^{2+} was due to the displacement of Ca^{2+} from intracellular sites, which then activated the K^+ channels.

The Ca^{2+} -activated K^+ channel of the hamster egg has been shown to be sensitive to Ca^{2+} and Sr^{2+} , but not Mg^{2+} or Ba^{2+} ; the effect of these divalent cations has been tested on hyperpolarisations at fertilisation (Igusa and Miyazaki, 1983), by iontophoretic injection (Georgiou, Bountra, McNiven and House, 1987) and during treatment with high pH solution (Georgiou, House, McNiven and Yoshida, 1988).

In channels reconstituted into planar lipid bilayers, neither Mg^{2+} , Ba^{2+} nor Cd^{2+} could activate a K^+ conductance (Latorre, Vergarra and Hidalgo, 1982). In contrast, using excised inside-out patches from pancreatic acinar cells, Iwatsuki^t and Petersen (1985) demonstrated a similar level of channel activation by Ba^{2+} (3.0 μM) and Ca^{2+} (1.0 μM). Higher concentrations of Ba^{2+} (1 mM) produced prolonged periods of channel closure at depolarised membrane potentials. This is similar to effects reported by Maruyama, et al (1983b) of channel closure at higher concentrations of Ca^{2+} at depolarised potentials.

McManus and Magleby (1984) performed an analysis of the kinetic properties of Ca^{2+} -activated K^+ channels from cultured rat muscle, using strontium as the activating cation. The distribution of channel opening and closing in the presence of 100 μM Sr^{2+} was similar to that observed in 0.5 μM Ca^{2+} . This would suggest that the decreased efficacy of Sr^{2+} in activating these channels is due to a lower forward rate constant for Sr^{2+} binding compared to Ca^{2+} , rather than from marked changes in channel kinetics.

Recently, activation of the Ca^{2+} activated K^+ channel by Mg^{2+}_i has been reported (Golowasch, et al, 1986; Squire and Petersen, 1987). In both cases, the presence of Mg^{2+} dramatically enhances the activation of the channel by Ca^{2+} in a dose-dependent way (see section on modulators of the

Ca²⁺-activated K⁺ channel). It would seem that Sr²⁺ is the only divalent cation that can activate the Ca²⁺-activated K⁺ conductance in most preparations.

In addition, it is of interest to note that strontium has been reported to support capacitation and the acrosome reaction in mouse sperm and that it rapidly activates mouse eggs (Fraser, 1987).

SECTION 2 General methods

An account of the general methods used in this study are presented here. Included are specific technical difficulties that arose and the attempts made to overcome them.

A Solutions

i. Normal solution.

The normal solution for bathing the hamster eggs was (mM): NaCl, 140; KCl, 5.5; CaCl₂, 1.7; MgCl₂, 1.2; glucose, 5.6 (from Igusa & Miyazaki, 1983). The solution was buffered with 10 mM HEPES (pK_a=7.5; 25 °C) and 10 mM CHES (pK_a=9.3; 25 °C) for the pH range 7.5 - 9.5 and with 10 mM MES (pK_a=6.1; 25 °C) and 10 mM HEPES for the pH range 5.5 - 7.5. These buffers are reported to have^{no} effects on the electrophysiological properties of membranes (Good, Winget, Winter, Connolly, Izawa and Singh, 1966), and no significant difference in the electrophysiological properties of hamster eggs was observed when CHES, HEPES or a combination of these was used to buffer the solution. Bovine serum albumin (Sigma) was added to all solutions (3 mg/ml).

When the concentration of Ca²⁺, K⁺, Mg²⁺, Ba²⁺ or Sr²⁺ was altered, the tonicity of the solution was maintained by adjusting the Na⁺ concentration. A list of the solutions used in the intracellular recording experiments is given in Table 1. Na⁺-free solutions were obtained by equimolar replacement of NaCl with N-methyl-D-glucamine (NMDG), and the pH adjusted with HCl. In the fertilisation experiments, Na⁺ was sometimes replaced by choline, and KOH used to titrate the solution to the correct pH value. Low Na⁺ solutions were obtained by equimolar replacement of 5/6 NaCl with LiCl.

Methane sulphonate (Aldrich) was substituted for Cl⁻ in some experiments to obtain Cl⁻-free solutions. It is a good substitute for several reasons: 1) it is impermeant, 2) its pK_a (-1.2) is far from the pH of the solution (7.5) so it is fully dissociated making equimolar replacement acceptable, 3) its use does not require a change in (Ca²⁺)_o (Sharp and Thomas, 1981). Ouabain and amiloride were obtained from Sigma while tetraethylammonium was obtained from BDH chemicals.

ii. Solutions used in the fertilisation experiments.

The normal solution bathing the hamster eggs during the fertilisation experiments was (mM); NaCl, 122.5; KCl, 5.5; CaCl₂, 4; MgCl₂, 1.2; glucose, 5.6 (from Igusa & Miyazaki, 1983). The solution was buffered with 10 mM HEPES (pKa=7.5; 25 °C) and 10 mM CHES (pKa=9.3; 25 °C) for the pH range 7.5 - 9.5 and with 10 mM MES (pKa=6.1; 25 °C) and 10 mM HEPES for the pH range 5.5 - 7.5. Na⁺-free solution was obtained by equimolar replacement of choline chloride for NaCl.

iii) Solutions used in the single channel recording experiments. In order to record the activity of Ca²⁺-activated K⁺ channels a high (K⁺) solution was used on both sides of the membrane patch; a reversal potential of 0 mV and high single channel conductance should be measured under these conditions. By omitting Na⁺ from the solution, contamination by Na⁺ channels is avoided. In addition, possible blocking effects of Na⁺ on the Ca²⁺-activated K⁺ channels (Yellen, 1984) are avoided. The buffer TES is reportedly satisfactory for these experiments (Blatz & Magleby, 1984).

Intracellular solution. The quantities of EGTA and calcium were chosen to give intracellular free Ca²⁺ concentrations equivalent to those reported for basal levels and those reported to activate Ca²⁺-activated K⁺ channels.

The solutions used in the single channel experiments are presented in Table 2.

	Normal	Na ⁺ free 1.7 Ca ²⁺	Na ⁺ free 20 Ca ²⁺	5/6 Li ⁺	Cl ⁻ free
NaCl	140	-	-	23.3	-
KCl	5.5	5.5	5.5	5.5	-
CaCl ₂	1.7	1.7	20	1.7	-
MgCl ₂	1.2	1.2	1.2	1.2	-
NMDG	-	140	112.6	-	-
LiCl	-	-	-	116.7	-
NaOH	-	-	-	-	140
KOH	-	-	-	-	5.5
Ca(OH) ₂	-	-	-	-	1.7
MgO	-	-	-	-	1.2
Methane					
Sulphonate	-	-	-	-	151.3
Glucose	5.6	5.6	5.6	5.6	5.6

Units mM.

Table 2.1 Solutions used for intracellular recording.

	0.1 μ M	0.5 μ M	4.0 μ M	Intrapipette
	free Ca ²⁺	free Ca ²⁺	free Ca ²⁺	solution
KCl	150	150	150	150
MgCl ₂	1.13	1.13	1.13	1.0
EGTA	1.1	1.1	1.1	-
CaCl ₂	0.55	0.135	1.08	5.0
HEPES	10	10	10	10

Units mM.

Table 2.2 Solutions for intracellular recording.

iii. Solutions for Capacitation of hamster sperm.

The composition of the solution in which sperm were isolated and capacitated was as follows (mM): NaCl, 124.8; KCl, 2.68; CaCl₂, 1.8; MgCl₂, 0.49; Na-monophosphate 0.36; Taurine, 0.5; Na-bicarbonate, 11.9; Na-pyruvate, 0.09; Na-lactate, 9.0; glucose, 4.5; L-adrenaline (Sigma) 0.05; Bovine serum albumin (Fraction V, Sigma) 3 mg/ml. (equilibrated with 5 % CO₂, 95% air; pH 7.4).

Once all components of the solution were added (except for the adrenaline and the albumin), the solution was 'bubbled' with a 5 % CO₂/95 % air mixture of gas to speed up the process of pH equilibration. The adrenaline and albumin were then added and the solution placed in an incubator which maintained the solution at 37 °C and the required CO₂ concentration.

B Collection of Gametes

i. Oocytes.

The egg donors were mature (6-8 weeks) virgin golden hamsters maintained under a controlled light-dark cycle (16 h light-8 h dark). They were induced to superovulate by first injecting them intraperitoneally (I.P.) with 30 i.u. of pregnant mares's serum gonadotrophin (PMSG) (Folligon; Intervet Labs.Ltd Cambridge) in the early evening followed 48 h later by an I.P. injection of 45 i.u. of HCG (chorionic gonadotrophin, ICG-2, Sigma Chemical Co, St Louis, U.S.A.).

Hamsters were killed by cervical dislocation 15-18 h after the HCG injection and their oviducts were dissected out and placed in a dish containing a physiological solution (referred to as normal, Table 1) at room temperature (20-22°C). The swollen tubal ampullae were torn with forceps and the cumulus mass squeezed out into the solution. The cumulus oophorus was removed enzymatically by bathing all the eggs for 1-2 minutes in normal solution containing hyaluronidase (1 mg/ml; Type 1-5, Sigma). They were then washed twice in normal solution and placed in an incubator at 37°C.

Whenever an egg was required it was transferred to normal solution containing trypsin (1 mg/ml, Type III, Sigma) for 30-60 s to remove its zona pellucida. The enzyme treatments were carried out at room temperature. Eggs were transferred in a 1 ml plastic pipette previously drawn out over a flame to give it a narrow diameter.

The eggs were observed with a microscope (x40) during trypsinisation and washed as soon as the zona pellucida appeared to come off the egg (indicated by a distortion of the shape of the egg).

Cumulus-oophorus free eggs occasionally underwent spontaneous activation during incubation for more than 3 h, and released the second polar body. Such eggs were discarded.

ii Sperm.

Male golden hamsters older than 10 weeks, weighing a minimum of 100 g, were used as sperm donors. Both cauda epididymi were dissected from the animal, then any fat and blood vessels were removed from their surface. The surface was further cleaned using filter paper moistened with double distilled water. The surface membrane of the cauda epididimis was cut, such that two or three of the sperm-filled tubules were exposed.

Each cauda epididymis was then placed near the edge of a sterile plastic dish, filled with liquid paraffin. Covered by the liquid paraffin was a drop of modified Tyrode solution (the composition of which is given above in A, iii). Both the modified Tyrode and the liquid paraffin had previously been equilibrated with 5 % CO₂ / 95 % air (Tyrode solution is buffered using Na-bicarbonate). The liquid paraffin limits evaporation of the solution in the incubator.

The dissection instruments were kept warm using a hot plate and/or an incubator, both being at 37°C.

A small nick was then made in one or two of the exposed sperm filled tubules, causing a tiny droplet of sperm to be released into the liquid paraffin. The size of this droplet (i.e. the number of sperm to be isolated) could be increased by applying gentle pressure to the cauda epididymis using a coarse pair of forceps. This droplet of sperm was then drawn into the droplet of Tyrode solution with a fine sterile glass hook. The sperm then swam freely away from the glass hook, to distribute themselves evenly within the Tyrode solution.

The concentration of sperm isolated into the droplet of Tyrode solution was then measured using a haemocytometer. Following this the sperm were then incubated at a concentration of 10⁻⁵ - 10⁻⁶ /ml for more than 3 h at 37°C, in an atmosphere of 5 % CO₂, 95 % air. This procedure is necessary to enable the sperm to undergo capacitation, a prerequisite for successful fertilisation (Austin, 1952).

Experiments were performed at 33-36 °C in a chamber heated by a coil or by circulating hot water, to decrease electrical noise. After the egg had been impaled with a recording electrode a small droplet of capacitated sperm suspension was added at the side of the chamber to the solution containing the egg. The final sperm concentration in the chamber was about 5000/ml. This relatively low concentration of sperm was chosen to minimise the number of sperm reaching the egg and to maximise the interval between sperm addition and sperm attachment to the egg.

Such fertilisation experiments were performed up to 18 hours after the injection of HCG. Zona-free eggs were used to avoid the delay which would otherwise be caused due to sperm passage through the zona pellucida. The time required for zona penetration in hamster eggs has been reported to be 4-22 minutes (Yang, Lin, Wang & Chang, 1972). Fertilisation of zona-free hamster eggs usually resulted in severe polyspermy, as observed by previous workers (Yanagimachi, 1972)

In both cases, an egg was placed in a chamber containing 3-5 ml normal solution, mounted on an inverted microscope (Nikon, Diaphot-TMD) inside a Faraday cage. The microscope was positioned on a heavy granite table to limit vibration artifacts. A 12 V car battery was used to power the light source, to reduce a.c. noise. Recording and injection micropipettes were introduced to the solution and placed in position near the egg using Narishige oil-driven micromanipulators.

Perfusion of the chamber was either performed by hand, with a pasteur pipette, or by constant perfusion system. In the latter case, solutions were pumped into and out of the chamber through silicon tubing, using a peristaltic pump set at a rate of about 10 ml per minute. When the solution bathing the egg required heating, a locally-made heating coil plus thermistor was placed in the chamber.

C Electrophysiological recordings

Two methods of electrophysiological recordings were used in this study and they will be described separately:

- 1 Conventional intracellular recording.
- 2 Single channel using the inside-out mode of operation.

1 Conventional microelectrode recording

a) Recording and injection micropipettes were made from capillary glass (Clark Electromedical Instruments; GC 200f-15 and GC150-15) and pulled on a locally-made electrode puller. A single microelectrode (40-80 M Ω) filled with 2 M potassium acetate was then inserted into the egg for simultaneous monitoring of membrane potential and input resistance. This microelectrode was connected to the input of a high impedance preamplifier (DAGAN 8100) set in the bridge mode of operation.

The electrode was inserted into the egg by over-compensation of the negative capacitance of the preamplifier. Current pulses from a Devices isolated stimulator (2533; Devices Ltd.) driven by

a digitimer (D4030; Devices Ltd) were passed between the barrel of the microelectrode and a bath ground (either an agar bath electrode containing 3 M KCl, or a silver-silver chloride wire).

In some experiments where action potentials were evoked by depolarising pulses, the rate of change of membrane potential was measured using an electronic differentiator.

Tape recordings on FM magnetic tape (Racal STORE 4DS) of the experimental data were routinely made, and permanent experimental records for reproduction were obtained as pen recorder traces on a Graphtec servocorder (SR 6335) or as photographs from the screen of a storage oscilloscope (RM 5113, Tektronix Ltd).

Single channel recordings were stored on video tapes using a conventional video cassette recorder (Panasonic) after being digitised by a Digital Audio Processor (Sony, PCM-701 ES) modified according to Lamb (1985) to accept d.c. current. Processing of this data is discussed under the section on single channel recording below.

b) EGTA injection. EGTA (Sigma) was injected from a second electrode inserted in to the egg. This electrode was filled with 0.25 EGTA (K salt) and had a resistance of 10-15 M Ω when filled with 3 M-KCl. The EGTA electrode was inserted into the cell in the same manner as the K acetate electrode. EGTA was applied intracellularly by iontophoresis or leakage from the EGTA pipette.

c) Ca²⁺ injection. Ca²⁺ was injected iontophoretically from a second electrode inserted into the egg. This electrode was filled with 1 M CaCl₂ and had a resistance of 10-15 M Ω s when filled with 3 M-KCl. The Ca²⁺ electrode was inserted into the egg in the same manner as the K-acetate electrode. Current pulses from a Devices stimulator were delivered to the Ca²⁺ electrode using a preamplifier (M 707; WPI Instruments, U.S.A.).

In some conventional intracellular recording preamplifiers (such as the WPI instruments M 707, the amount of current injected is not directly measured. Instead, the voltage pulse from the stimulator applied to the preamplifier to inject the calcium is measured at the 'stimulus in' position. A problem then arises if the injection-electrode resistance increases; calcium electrodes are prone to this as they block easily. The recorded voltage pulse suggests that the same amount of calcium is being injected for a given voltage pulse; depending on the extent of pipette blocking, a large variation in this may occur (an example of this is illustrated in Fig 1A).

A more satisfactory method of measuring the current injected into the egg is to monitor the current flow through the egg directly with a virtual ground (current-to-voltage converter). If current is injected into the egg through a calcium electrode, and the actual current is directly measured, the

current is often irregular; it does not follow the applied rectangular voltage pulse, as would be recorded by the preamplifier. Instead, it appears like the example in Fig 1B. The quantity of charge passed through the calcium electrode was estimated by measuring the area under this pulse.

It is acknowledged that even this is not an ideal situation, as charge might not be carried solely by Ca^{2+} ions. This method was used in Section 3 in order to ensure that comparisons were made between responses with the same size of current pulse. The virtual ground circuit thus performs two functions, first in providing a stable ground level unaffected by current, and secondly, as a means of measuring current directly.

The quantity of charge passed through the Ca^{2+} electrode was estimated by measuring $\int I dt$ from traces of I vs time (t) (like the example in Fig.1B) because the shape of the current was not always rectangular, due to blockage of the Ca^{2+} electrode. A disadvantage of the virtual ground system is that it is noisier than the current-measuring resistor system in some conventional preamplifiers. This is not an important consideration for experiments involving intracellular microelectrode recordings, where the currents are quite large.

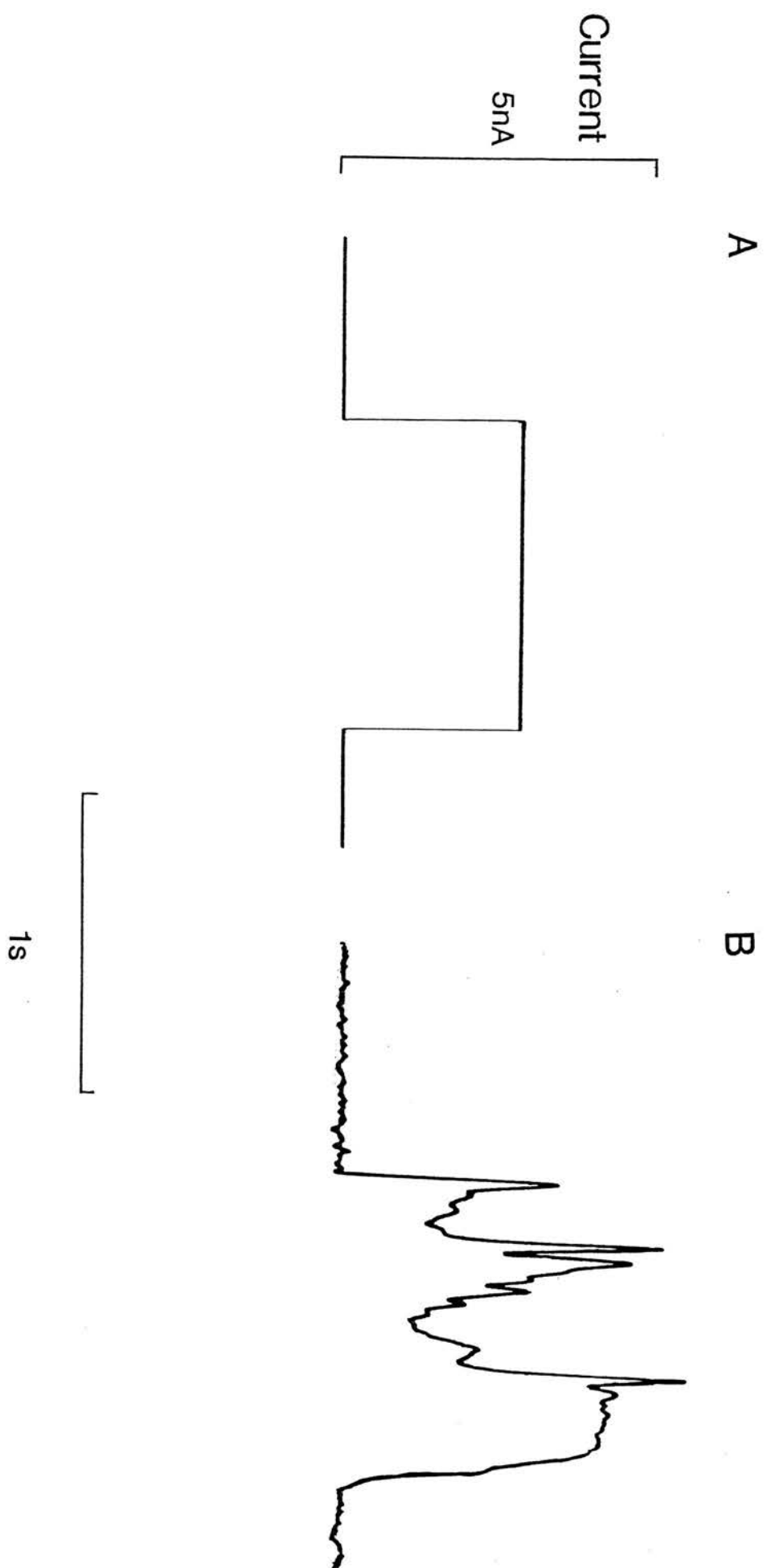


Figure 2.1. Two methods of measuring the size of the current pulse injected into the egg through a calcium micro-pipette. **A.** This is the current recorded directly from the preamplifier (actually just a measure of the voltage pulse applied to the preamplifier). **B.** This is the same current pulse recorded using a virtual ground system. It shows the actual current pulse passing through the egg to earth.

d) Reversal potential estimation. The reversal potential of fluctuations in the egg membrane potential was estimated according to the method described by Ginsborg, House & Silinsky (1974), using the equation:

$$E_R = (P/P-p)v + E_M$$

where E_R is the reversal potential of the response, E_M is the resting potential, P is the amplitude of electrotonic potential at rest, p is the amplitude of the electrotonic potential at the peak of the response and v is the amplitude of the potential change from the resting potential to the peak of the response. This equation is only valid if the resting conductance is independent of the applied current and hence of the value of the membrane potential. Therefore the strength of the current pulses used was such that the resulting electrotonic potentials fell within the linear region of the current-voltage relation.

e) Intracellular pH measurements.

Measurements of intracellular pH were made using pH-sensitive micropipettes. These were made by treating the tip of a micropipette with ^{the} ~~silane~~ ^{tri-n-butyl chlorosilane} to make them hydrophobic. They are then filled with an organic solution sensitive to pH. The pH-sensitive micropipettes were inserted into the hamster egg by gently tapping the micromanipulator (Thomas, 1978).

The electrical set-up used to measure pH_i was a conventional one; one locally-made high impedance amplifier for the pH-electrode and another for a conventional microelectrode (needed to serve as a reference for the pH electrode). This second micropipette was inserted into the egg by over-compensation of the negative capacitance of the amplifier. The pH electrode was calibrated by bathing it with solutions of different pH.

2 Single Channel Recording

The inside-out patch conformation of the single channel recording mode was used; the initial procedure involved a high resistance seal (1-3 GΩ) being formed between the patch-electrode and the egg membrane, isolating a patch of membrane. A number of procedures were necessary to obtain this high resistance seal.

a) Treatment of the egg. The enzyme treatment for the egg was extensive in single channel recording, in an attempt to expose the membrane surface, allowing a tighter seal between the patch-electrode and the egg membrane. After the egg was removed from the cumulus oophorus with hyaluronidase (see above) it was bathed in a solution of 1 mg/ml protease (Sigma) for 5-20

minutes at 37 °C. It was then carefully washed in an albumin-free solution and placed in the chamber (albumin particles prevented tight sealing between electrode glass and the egg membrane).

The composition of the solution in the chamber and that in the patch-electrode was the same (see Table 2) to eliminate contamination of either, or the production of a junction potential. These solutions were all filtered to remove any particles that might reduce sealing resistance. After the high-resistance seal was obtained, albumin-containing solution was added to the chamber to stabilise the membrane (in the cell-attached configuration). It was unnecessary to filter this solution after the Giga-seal was obtained.

b) Cell-attached mode. The patch-electrode was placed in the solution and the resistance monitored by current pulses from a Devices isolated stimulator (2533; Devices Ltd.) driven by a digitimer (D4030; Devices Ltd) passed between the barrel of the patch-pipette and a silver-silver chloride ground electrode or an agar ground. The typical resistance of the patch-pipette was 2-5 M Ω , with a tip diameter of 0.5 μ M, calculated according to the method of Mittman, Flaming, Copenhagen and Belgum (1987), described below. The pipette was then gently lowered on to a zona-free egg. The resistance usually rose to 100-200 M Ω . A giga-seal was then obtained by applying negative pressure to the back of the pipette via an outlet on the probe using a 1 ml syringe. This pressure was monitored using a water-column connected to the syringe creating the negative pressure.

Some variation in the time taken for Giga-seal formation was observed; from 10 s to 20 min. This could reflect variation in the egg-surface topography, susceptibility of the zona pellucida to the enzyme used or variations in tip geometry.

c) Inside-out patch mode. Once a Giga-seal was attained, it was possible to excise a patch of membrane from the egg. This was done by replacing the solution in the bath for one mimicking the intracellular solution, with a low calcium concentration (see Table 2). By pulling the patch-pipette quickly away from the egg, a patch of membrane was removed. The membrane was then exposed to the air for 1 or 2 s to avoid membrane vesicle formation. Here, what was the intracellular surface of the membrane now faces the chamber solution, and the extracellular surface faces the intra-pipette solution (Hamill et al., 1981). The advantage of the inside-out mode is that the 'intracellular' environment can be manipulated.

d) Patch-pipettes. The patch-pipettes were made from Garner glass capillary tubes. These pipettes were pulled on a Brown-Flaming microelectrode puller. This puller can be set to give a jet of nitrogen gas at a crucial point in the pulling of the pipette. This produces patch-pipettes with one pull (a range of resistances are available) that do not require fire-polishing, usually a prerequisite to obtaining a Giga-seal (Sakman & Neher, 1983).

The possibility of low channel density in these eggs dictates a compromise in the size of the patch-pipette. If the pipette-tip diameter was too small, the probability of finding a channel under the patch was very low, while if the pipette-tip diameter was too large more than one channel occurred in the patch, making single channel analysis difficult.

After pulling, a rough estimate of the resistance of the patch-pipette could be obtained, which is an index of the tip diameter. A 1 ml syringe was attached to the patch-pipette and the tip submerged in absolute methanol (Sigma). Pressure was applied to the pipette by syringe plunger until tiny air bubbles leave the tip of the pipette. The gradations on the side of the syringe provide the 'bubble number', the value where air bubbles start to come out of the pipette. The methanol evaporated quickly after the pipette is removed, and the pipette prepared for use if suitable.

The pipette was filled by dipping the tip in the required solution and applying suction to the other end with a 1 ml syringe. Care was taken to release the pressure before removal of the tip from solution, preventing bubble formation. The pipette was then back-filled by injecting solution through a fine tube. The back end of the pipette was held over a flame until a centimetre or two of solution was spat out. This prevents solution from entering the probe head, causing stray capacitance when the patch-pipette is in position .

Occasionally, electrodes were coated with Sylgard, a hydrophobic silicone elastomer. This reduces capacitance to ground due to the solution creeping up the exterior of the patch-pipette .

e) Data Processing. The sampling frequency of the digital audio processor was 44.1 kHz, and the raw data were recorded at this frequency for storage. Single channel data were filtered with a locally-made 8 pole Bessel filter, at 500 Hz for determining single channel current amplitude and 1.5 kHz for analysis of open- and closed-channel kinetics.

The data were digitised with an interface (locally-made) and then analysed using a patch clamp analysis program, PAT-V33 developed by Mr J. Dempster (Department of Pharmacology, University of Strathclyde).

Single channel current amplitude was measured either by hand or from a current amplitude histogram assembled by PAT-V33. Open-and closed-channel durations were measured by using an open-closed state transition detection method. A state was defined as the time between two open-closed or closed-open transitions.

The threshold for a state transition was set at 50 % (Colquhoun and Sigworth, 1983). During transition detection, an idealised trace was displayed, in conjunction with the experimental data trace, indicating at which points the program was detecting transitions. Subsequently, each open

and closed state in the detection list was viewed by eye and either accepted or rejected for inclusion in the lifetime analysis.

The distributions for open- and closed-channel durations were fitted with a multi-exponential probability density function (p.d.f.) using a Levenberg-Marquardt, non-linear least-squares method (SSQMIN, applied first by Dionne and Leibowitz, 1982). Each p.d.f. provided the value of the decay time constant for the exponential and the corresponding area under the distribution curve.

The advantage of using a pulse code modulator (PCM) and video system is that FM tape records analog signals. Limits are set by the mechanical characteristics of the tape and machine heads. They cannot follow a wide dynamic range, the frequency response is bad and signal distortion is a problem. The signal-to-noise ratio is much higher for video than FM tape. The disadvantage of using the PCM is that it was developed to accept a.c. signals. It needs to be modified to accept d.c. signal, which introduces a problem with d.c. drift (this is a temperature-related problem, and is solved by leaving the PCM to warm up).

f) Improved ground system. In Britain, the ground system for electrophysiology machines is usually provided by the third (earth) pin of the power cable plugged into the mains. This allows for the possibility of ground loops when the recording machines are wired up.

A more satisfactory system is to use a central ground board. In this case the third (earth) pin connection is removed from the plug. The machines are then grounded through a connection from the chassis ground to the rack they are mounted on. A single shielded wire runs from the rack to a low resistance central ground board. This board consists of a thick copper wire with many connectors. The central board is then grounded to a single ground (either the mains ground or a thick copper rod driven into the earth. Ideally, the central ground board should be as close to the most important recording machine as possible (usually the oscilloscope, tape recorder or PCM).

g) Junction potentials were measured by perfusing the chamber with the various solutions used to investigate the channels in the hamster egg. These were subtracted from the values recorded when the solutions were changed with the egg membrane present.

SECTION 3 Injection of Ca^{2+} and other cations into the unfertilised hamster egg.

In order to investigate Ca^{2+} -activated K^+ channels using conventional intracellular recording, it is necessary to activate them by raising the intracellular Ca^{2+} concentration. One method of doing this is to inject Ca^{2+} iontophoretically into the egg. This has been reported to produce a transient membrane hyperpolarisation associated with a marked reduction in the egg's input resistance (Miyazaki & Igusa, 1982; Igusa & Miyazaki, 1983; Georgiou et al, 1983). An example of a hyperpolarisation evoked by Ca^{2+} injection is illustrated in Fig.3.1.

There are several problems associated with Ca^{2+} injection, firstly in quantifying the amount of Ca^{2+} injected, and secondly in assessing the possible damage to the egg associated with the injection process. Previous data suggested that occasionally the iontophoretic pulse, in addition to injecting Ca^{2+} , also caused membrane break-down (Georgiou, Bountra, McNiven and House, 1987). This could result in an additional ionic pathway through the membrane.

The results presented in this Section forms an investigation of the latter problem; the question of quantifying the injected Ca^{2+} is dealt with in the Section 2. In addition, the effects of various chemicals on the passive electrical properties of the hamster egg are described here; these chemicals are reported to inhibit Ca^{2+} extrusion systems in other preparations. The work in the following Section combines the iontophoretic injection of Ca^{2+} and application of these treatments capable of inhibiting Ca^{2+} extrusion, so it was necessary to investigate the effects of these treatments on their own as control experiments.

Results

A Effect of the iontophoretic pulse on the membrane of the egg.

A hyperpolarisation of the hamster egg membrane (Fig. 3.1), due to activation of Ca^{2+} -activated K^+ channels, was obtained with a brief (1 s) depolarising current pulse (5-6 nA) passed through the Ca^{2+} injection pipette. This size of pulse also produces a displacement of the membrane potential of about +150 mV for the duration of the pulse. It was possible that such large potential excursions might cause the hyperpolarising responses by a non-selective mechanism ('punch through', Coster, 1965) rather than by Ca^{2+} entering the egg from the pipette.

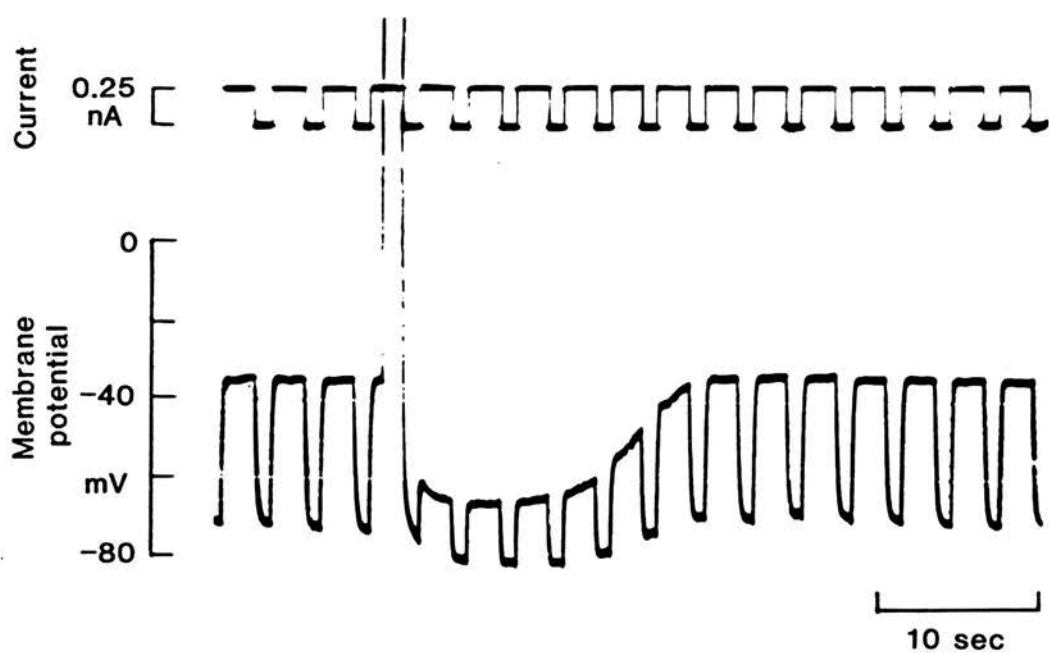


Fig.3.1. An example of membrane hyperpolarisation and fall in input resistance evoked by iontophoretic injection of calcium into a hamster egg from a micro-pipette containing 1 M calcium chloride. The lower trace is the membrane potential of the egg, recorded by an intracellular microelectrode. The brief downward deflexions on the lower trace are electrotonic potentials resulting from current pulses passed through the recording microelectrode. The upper trace is a record of these current pulses. From these the input resistance of the egg may be monitored. The large positive deflexion of the membrane potential prior to the hyperpolarisation is due to the injection of calcium through the calcium micro-pipette.

Membrane Potential

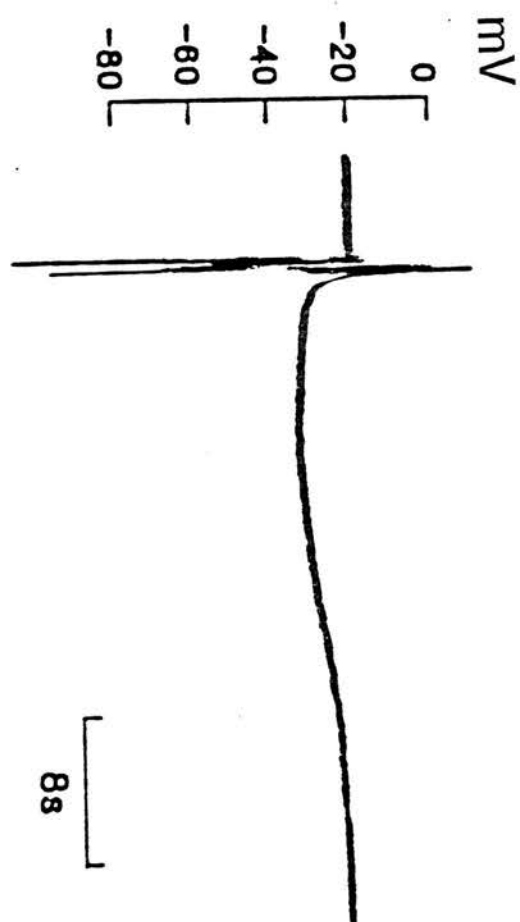


Figure 3.2. A variation on the technique for injection of calcium into hamster eggs shows the response of another egg to injected calcium, without electrotonic potentials present. Here, the depolarisation produced by the current pulse passed through the calcium-filled micropipette has been somewhat offset by a corresponding hyperpolarising current pulse passed through the recording micro-electrode.

To examine this possibility three types of experiments were performed. In the first type of experiment (n=4), a hyperpolarising pulse was passed through the recording electrode in an attempt to counter-balance the depolarisation caused by current injection through the Ca^{2+} pipette. Illustrated in Fig.3.2 is a representative example of this, where the bulk of membrane potential displacement produced by the two pulses almost cancel one another out. The resulting hyperpolarisation is not identical to one obtained without a counterbalancing pulse. However, it is difficult to determine the amount of Ca^{2+} entering the cell under these conditions.

In the second group of experiments (n=5), instead of a pulse with a large amplitude and short duration, small current pulses were applied for a longer time. For example, pulses of about 5 - 15 s duration were passed through the Ca^{2+} pipettes causing depolarisations of about 20 mV only, evoking graded hyperpolarisations (Fig. 3.3). When the injection pipette was filled with 1 M-NaCl and prolonged current pulses passed through it, transient hyperpolarisations were not produced (n=4).

Finally, in the third type of experiment, iontophoretic injection of ions other than Ca^{2+} were made. Iontophoretic injection of K^+ (n=2), Mg^{2+} (n=4), Co^{2+} (n=3), Ba^{2+} (n=3) or La^{3+} (n=2) failed to evoke a hyperpolarisation but instead often caused a depolarisation with concomitant fall in the cell's input resistance with slow recovery.

Representative examples of such 'break-down' responses produced by the iontophoretic injection of Mg^{2+} are shown in Fig 3.4. All the responses were obtained from the same egg whose membrane potential was changed to different values by injecting d.c. current. Pen traces of the responses are shown on the left of the figure. The faster records on the right are oscilloscope pictures corresponding to the responses displayed on the left. The reversal potential of these responses was -7 mV since at that value the membrane potential did not appreciably change after the Mg^{2+} injection despite a prominent and long-lasting fall in the input resistance (Fig. 3.4B).

The effect of the iontophoretic pulse when the membrane potential was shifted to -40 mV was a depolarisation which was associated with a large reduction in the cells input resistance (Fig.3.4C). In direct contrast, a hyperpolarisation was observed in response to the injection of Mg^{2+} when the membrane was shifted to a value of +20 mV (Fig. 3.4A). The measured value of the rise in the input conductance of the responses illustrated in Fig.3.4 was 7.3 ± 2.5 nS.

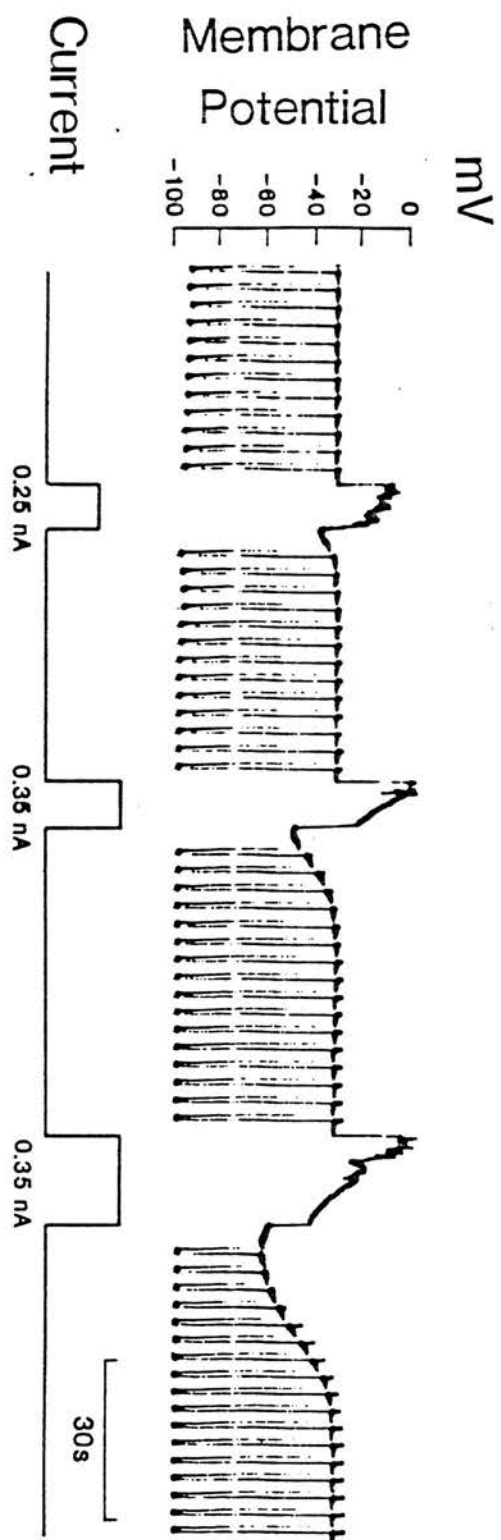


Figure 3.3. An illustration of responses of a hamster egg to prolonged, small current pulses passed through the calciumfilled micro-pipette. The iontophoretic current pulses passed through the calcium micropipette are shown below the recording of the membrane potential. Electrotonic potentials were not applied to the egg for the duration of the calcium pulse.

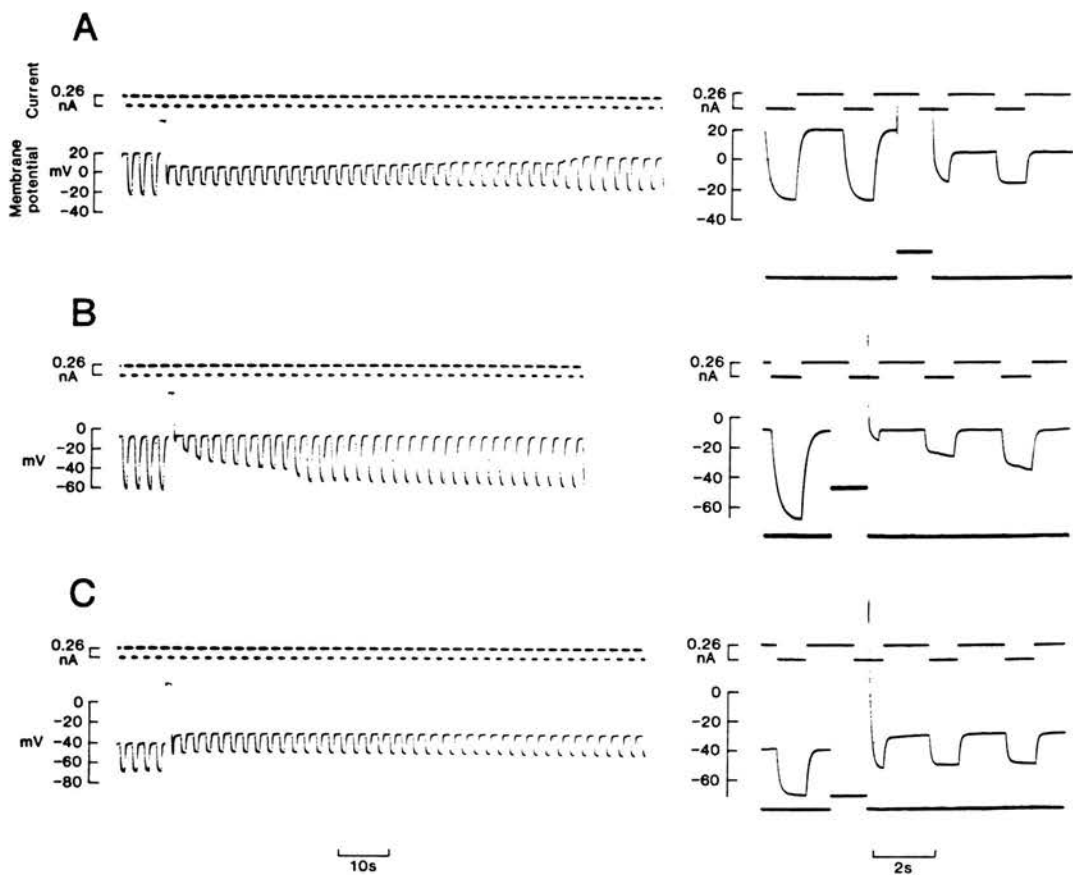


Fig. 3.4. 'Break-down' responses elicited by the iontophoretic injection of magnesium. The downward deflexions of the membrane potential recordings were produced by the passage of constant-current pulses (shown above each membrane potential trace) through the recording micro-electrode. The rectangular deflexion indicated below each membrane potential trace on the right hand side of the Figure is the current pulse applied to the magnesium pipette.

In six other similar experiments a wide variation in the value of the rise in the input conductance was observed ranging from 1 to 55 nS. The corresponding value of the reversal potential was -11 ± 5 mV. This suggests that the injection-induced leakage current is not selective towards any particular ion. The time taken for the resistance to recover to its original value after such injections was variable. An attempt was made to quantify this recovery with respect to time; the percentage recovery of input resistance was calculated from the following expression;

$$(R_0 - R_t / R_0) 100 = \% \text{ recovery of input resistance.}$$

where R_0 is the value of the input resistance just before the electrophoretic Ca^{2+} pulse, and R_t is the input resistance at a time, t , after the pulse. The recovery of the membrane input resistance followed an exponential time course; a semilogarithmic (\log_e) plot of percentage fall in input resistance against time was linear (as illustrated in Fig. 3.5). The time constant, T_R , for this recovery of input resistance is given by the inverse of the slope.

A large variation in T_R was obtained, ranging from 13 to 160 s. The larger values, indicating a long time for recovery, were associated with larger current pulses. These larger values of T_R observed for the Mg^{2+} -evoked responses, e.g. 160 s, were similar to the values obtained for responses to Ca^{2+} -injection with reversal potentials more positive than -70 mV by Georgiou et al (1987).

B Iontophoretic injection of Sr^{2+} .

Iontophoretic injections of strontium into hamster eggs mimicked the action of Ca^{2+} by producing a transient hyperpolarisation and fall in resistance. The calculated value of the reversal potential of the Sr^{2+} -evoked responses was -78 ± 7 mV ($n=7$). The rise in input conductance (g_K) measured at the peak of the response was 12 ± 9 nS ($n=7$). The value of T_R was 17 ± 14 s. The above values of reversal potential and g_K are comparable to those of Ca^{2+} -evoked responses with short recovery time constants of the input resistance, where $T_R = 12 \pm 7$ s, $n=41$. The corresponding values of reversal potential and g_K for previously reported Ca^{2+} -evoked responses were -77 ± 5 mV and 16 ± 8 nS respectively ($n=41$) (Georgiou et al, 1987).

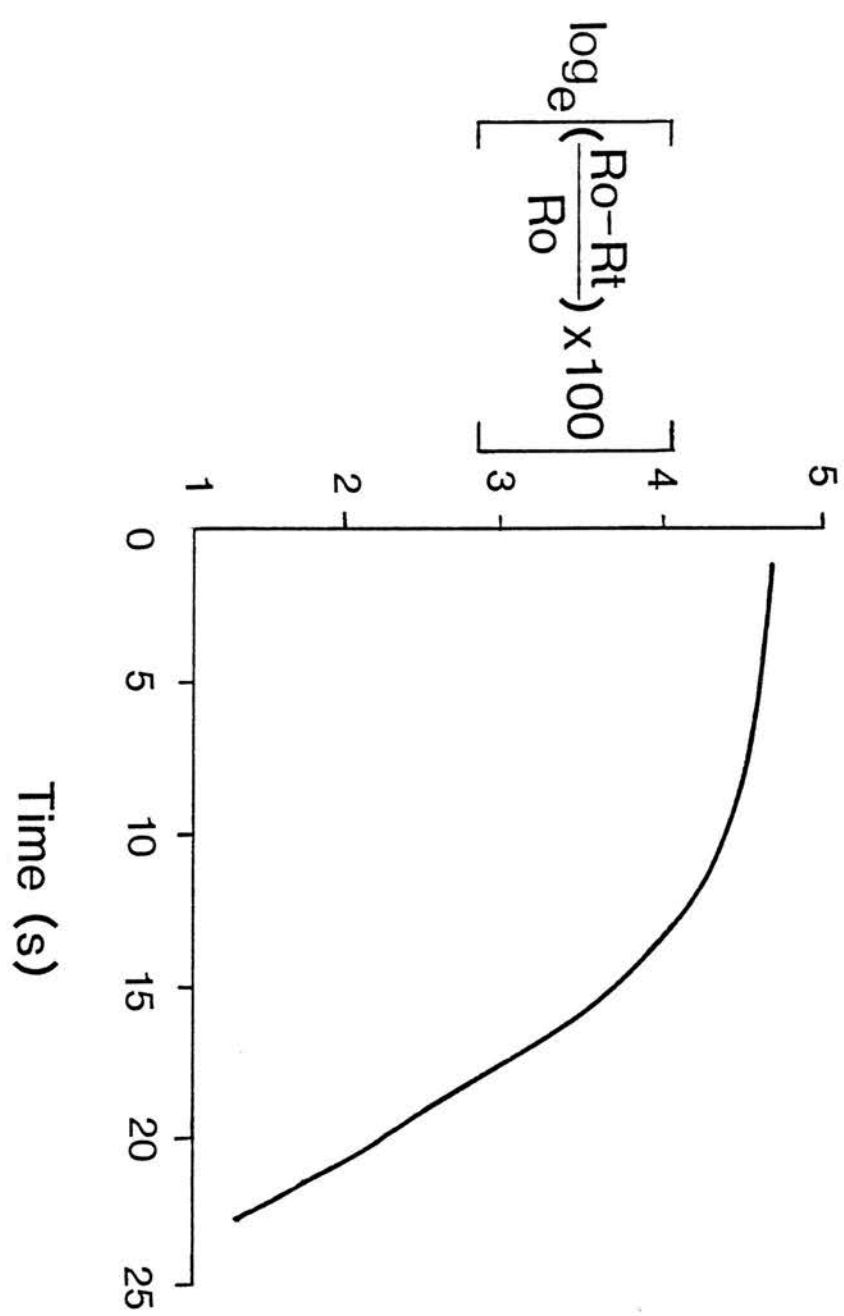


Figure 3.5. The recovery of input resistance with time after iontophoretic injection of magnesium ions. The values R_o (input resistance before the magnesium injection) and R_t (input resistance at a time 't' after the magnesium injection) are calculated from electrotonic potentials.

C The effects of Ca^{2+} , La^{3+} and DNP on the passive electrical properties of the egg.

In the experiments on the time course of the Ca^{2+} -evoked hyperpolarisation discussed in Section 4, it was observed that extracellular Ca^{2+} , lanthanum and DNP had effects on the passive electrical properties of eggs. Those effects have been discussed separately here for the sake of clarity.

The effect of raising the external Ca^{2+} concentration from 4 to 20 mM was a steady and reversible shift in the egg's membrane potential from -20, -18 and -30 mV to the values of -24, -26 and -33 mV respectively ($n=3$). These shifts were accompanied by corresponding rises in the egg's input resistance from the values of 150, 62 and 270 $\text{M}\Omega$ to the values of 200, 94 and 350 $\text{M}\Omega$.

Bath application of 1 mM La^{3+} consistently produced an increase in the membrane potential from a value of -27 ± 6 mV ($n=13$) before lanthanum treatment to a value of -44 ± 10 mV ($n=13$) during lanthanum treatment. This hyperpolarising shift was always associated with a corresponding rise in the egg input resistance from a value of 220 ± 130 $\text{M}\Omega$ ($n=11$) to a value of 390 ± 140 $\text{M}\Omega$ ($n=11$). The effect of lanthanum on membrane potential and resistance was fully reversible

In eleven experiments, bath application of DNP at a concentration of 0.2-2 mM produced a steady membrane depolarisation (from a value of -22 ± 10 mV in normal solution to a value of -13 ± 7 mV in DNP-containing solution). The depolarisation was always accompanied by a substantial fall in the egg input resistance (from a value of 196 ± 134 $\text{M}\Omega$ before to a value of 120 ± 68 $\text{M}\Omega$ after exposure to DNP). The DNP effect was also fully reversible but required at least 10 min washing.

Discussion.

Despite the difficulties associated with iontophoretic injection of Ca^{2+} , it is a useful method of studying activation of Ca^{2+} -activated K^+ current, providing certain precautions are taken; the injection current should not be too high, or else damage to the membrane may occur.

The Mg^{2+} injection results suggest that such iontophoretic pulses may induce membrane break-down, judging by the recovery profile of input resistance in the Mg^{2+} experiments reported here and the Ca^{2+} -injections performed by Georgiou et al (1987). There was a large scatter in the value of T_R observed in these responses to Ca^{2+} injection with low reversal potentials.

Considering the magnesium injection results, an explanation for this scatter may be that the Ca^{2+} pulse could also cause membrane break-down with various rates of recovery. The rate of recovery for Ca^{2+} -evoked responses with high reversal potentials (more negative than -70 mV) was much faster, suggesting that membrane damage was minimal. This is because a shift in the reversal potential away from the equilibrium potential for K^+ would be expected if damage of the membrane occurs, due to other ions such as Na^+ passing through the membrane. As Mg^{2+} does not evoke a specific Ca^{2+} -conductance, the time taken for the input resistance to recover was probably due to breakdown of the membrane and a non-specific (or 'leak') pathway opening up. In this respect, the Ca^{2+} -evoked responses with positive reversal potentials may have a similar component to the conductance induced by Ca^{2+} . Thus, in experiments in Section 4, where the effect of various chemicals on the duration of Ca^{2+} -evoked responses was investigated, comparisons between responses with a high reversal potential were made. These were less likely to be affected by membrane break-down.

The results of the experiments where displacement of the membrane potential was minimal suggest that iontophoretic Ca^{2+} injection need not lead to breakdown of the membrane; the recovery of the input resistance in these experiments is comparable with that of Ca^{2+} injection experiments where the reversal potential was near the equilibrium potential for K^+ .

Previous workers had shown that Ca^{2+} -activated K^+ channels in other preparations could be activated by intracellular Sr^{2+} (Meech, 1978). A single channel study in cultured rat muscle by McManus and Magleby, (1984) indicates that a much higher concentration of Sr^{2+} than Ca^{2+} is required to activate Ca^{2+} -activated K^+ channels to the same extent. Latorre, Vergara and Hidalgo (1982) found that neither Ba^{2+} nor Mg^{2+} could activate this conductance in channels reconstituted from rabbit skeletal muscle. It was therefore of interest to determine if other cations than Ca^{2+} could activate the K^+ conductance of the hamster egg. The egg membrane hyperpolarisation was evoked by injection of Ca^{2+} or Sr^{2+} ions but not by Mg^{2+} , Ba^{2+} , Co^{2+} or La^{3+} .

This is an interesting result, as Igusa and Miyazaki (1983) report that extracellular strontium can sustain the Ca^{2+} -activated K^+ conductance underlying the repetitive hyperpolarisations seen in fertilisation of hamster eggs. They noted that it is less effective than Ca^{2+} at sustaining hyperpolarisations. Likewise, Georgiou, House, McNiven and Yoshida (1988) demonstrated that although Sr^{2+} could sustain the activation of Ca^{2+} -activated K^+ channels in the hamster egg by high extracellular pH, a higher concentration was required than for Ca^{2+} .

The effects of La^{3+} and high Ca^{2+} on the passive electrical properties of the hamster egg may be attributed to an improved sealing of the micro-pipette glass to the egg membrane. This would decrease the non-specific leakage pathway around the electrodes, leading to an increase in input



resistance and a hyperpolarisation, the result observed. However, the ability of 1 mM La^{3+} to match 20 mM Ca^{2+} in producing such ~~a~~ ^{is} an effect ~~is~~ a little surprising, when considering the stabilising action of these ions (Okamoto, Takahashi and Yamashita, 1977). It may be that La^{3+} acts as a blocker of Na^+ channels; this may remove the contribution of a Na^+ current from the resting potential of the cell, producing a hyperpolarisation and increase in input resistance that way.

DNP was seen to cause a depolarisation in the resting potential of the egg. It has been reported to cause membrane depolarisation in squid axons (Boron & De Weer, 1976) but no explanation was offered for its mode of action. DNP can carry hydrogen ions across cell membranes and it may be this feature that produces the depolarisation in hamster eggs; another protonophore, carbonyl cyanide m-chlorophenylhydrazone is reported to cause depolarisations of the plasmalemma of thallus cells of the aquatic liverwort *Riccia fluitans* (Felle & Bentrup, 1977); the depolarisation was associated with a rise in membrane conductance. Felle and Bentrup attributed the conductance rise to a massive increase in the permeability ratio $P_{\text{H}^+}/P_{\text{K}^+}$. However, recordings of pH_i in the hamster egg (Section 5) suggest that pH_i is more acidic than pH_o . It is difficult to see how an increase in P_{H^+} would lead to a depolarisation; the gradient for H^+ would cause an efflux of H^+ under these conditions, hyperpolarising the egg.

SECTION 4 Investigation of the Ca^{2+} -evoked hyperpolarisation

The results presented in this section form an investigation of the time-course of the transient membrane hyperpolarisation induced by Ca^{2+} injection (see Section 3). As a Ca^{2+} -mediated K^+ conductance underlies this hyperpolarisation, altering the rate of Ca^{2+} extrusion might alter the duration of the hyperpolarisation.

The effect of inhibitors of Ca^{2+} extrusion on the Ca^{2+} -evoked hyperpolarisation was therefore examined. Some of these inhibitors had effects on the passive electrical properties of the hamster egg and these are discussed in Section 3.

Results

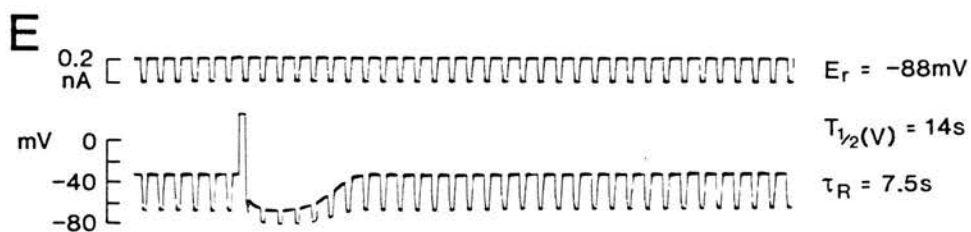
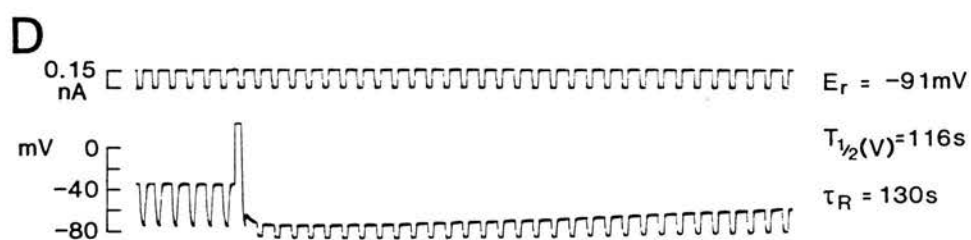
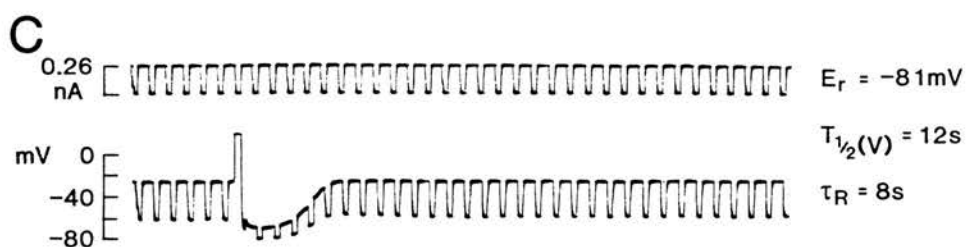
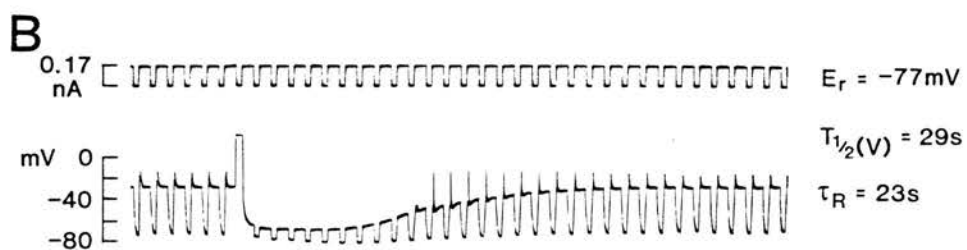
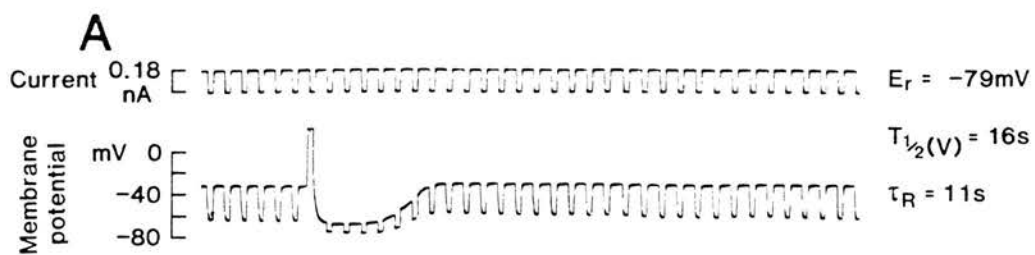
A Prolongation of the Ca^{2+} -evoked hyperpolarisations by high Ca^{2+} .

A high extracellular Ca^{2+} concentration has been reported to inhibit the Ca^{2+} -ATPase in squid axons (Dipolo & Beage, 1982). Iontophoretic Ca^{2+} injections in the presence of high external Ca^{2+} (20 mM) produced hyperpolarising responses which were considerably longer than responses obtained in 4 mM external Ca^{2+} . Fig. 4.1 shows representative examples of Ca^{2+} -evoked responses elicited in an egg bathed in normal (A), 20 mM Ca^{2+} (B) and back in normal solution following recovery from exposure to high Ca^{2+} (C). Indicated adjacent to each response are the values of the duration of the response at half its amplitude $T_{1/2(V)}$, the time constant of the recovery of the input resistance (T_R) and the value of the reversal potential (E_R). Fig. 4.2 illustrates the method for calculating the values of $T_{1/2(V)}$, $T_{(V)}$ and T_R . The method for calculating T_R is described in Section 3.

The reversal potential was calculated as described in Section 2. The reversal potential was not appreciably altered by the high Ca^{2+} treatment, thus excluding the possibility that T_R was extended as a result of iontophoretically-induced membrane break-down, as suggested for the results of MgCl_2 injection (see Section 3). The parameter $T_{1/2(V)}$ was chosen to enable comparison of the responses in the presence of the various inhibitors (the plateau phase of the DNP-induced prolongation made it difficult to measure the parameter $T_{(V)}$, which requires an obvious peak amplitude of the response).

As shown in Fig. 4.1, the prolongation of the potential change was always associated with a corresponding prolongation in T_R . Since the value of T_R could be over-estimated, given that the

membrane might suffer damage during the iontophoretic Ca^{2+} pulse, comparisons were made between the responses with high reversal potentials. In five experiments the value of the reversal potential of responses obtained in normal, high Ca^{2+} and normal (recovery) solutions was -77 ± 8 , -80 ± 8 and -74 ± 6 mV ($n=5$) respectively. In two further experiments the Ca^{2+} micro-electrode dislodged itself from the egg before recovery could be achieved.



30s

Figure 4.1 The prolonging effects of high calcium and lanthanum on calcium-evoked responses. Responses A, C and E were obtained in normal solution whereas responses B and D were obtained in the presence of high calcium (20 mM) and La^{3+} (1 mM) respectively. The order in which the responses were obtained corresponds to the sequence of labelling. The downward deflexions of the membrane potential records are electrotonic potentials elicited by the application of constant-current pulses (shown above each membrane potential record) through the recording microelectrode.

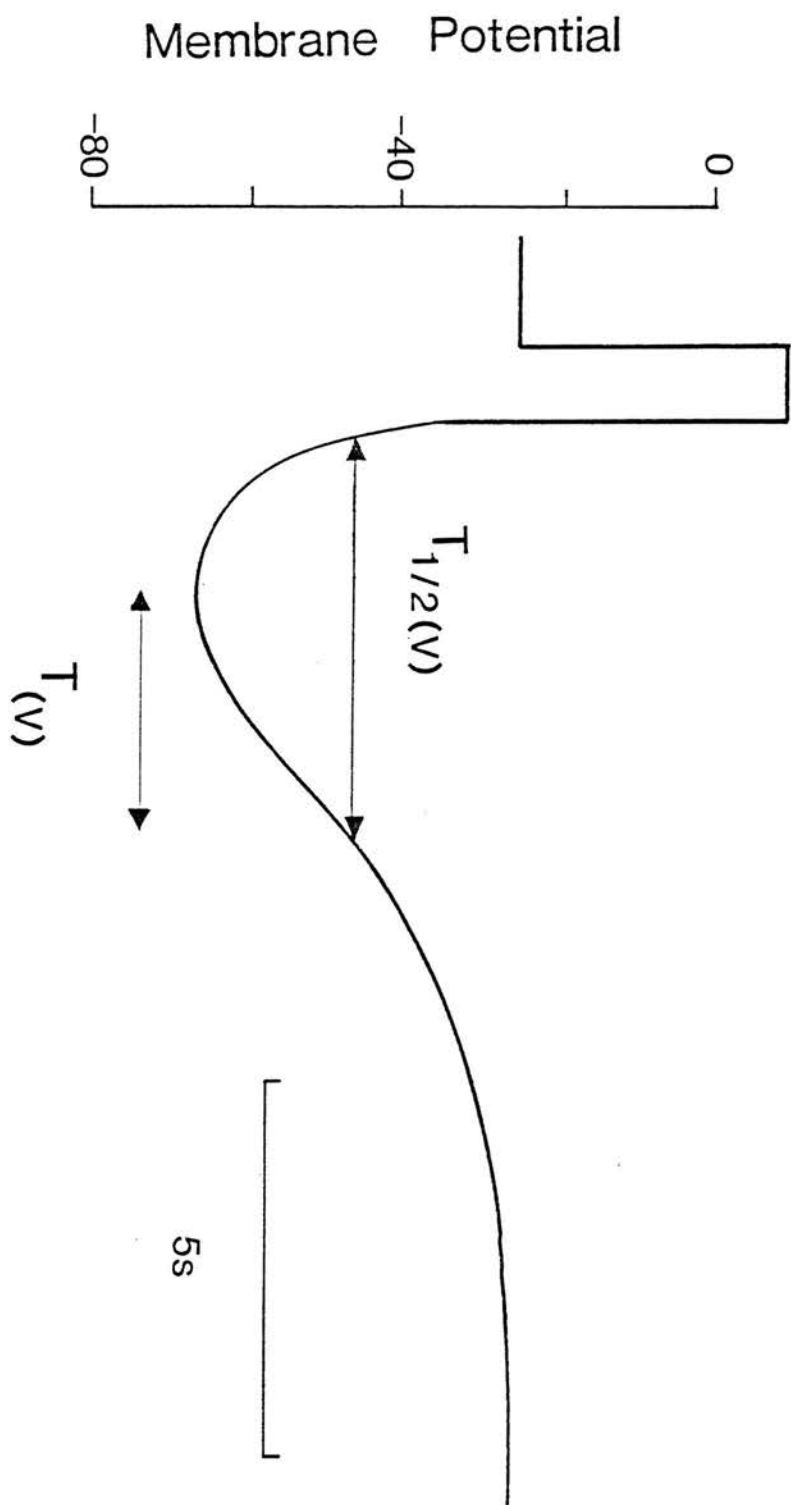


Figure 4.2. Diagram showing positions for measurement of parameters used to measure the duration of a hyperpolarisation evoked by iontophoretic calcium injection. $T_{1/2(v)}$ is the duration of the response at half its maximum amplitude. $T_{(v)}$ is also the duration of the response at half its maximum amplitude, but measured from the peak of the response.

The measured values of $T_{1/2(V)}$ for the responses in normal, 20 mM Ca^{2+} and normal (recovery) solutions were 20 ± 14 , 60 ± 14 and 17 ± 7 s respectively ($n=5$). The corresponding values for T_R were 15 ± 9 (normal), 23 ± 13 (20 mM Ca^{2+}) and 12 ± 3 s (recovery, $n=5$). A paired t test showed that the observed mean increases of $T_{1/2(V)}$ and T_R in responses obtained in 20 mM compared to 4 mM Ca^{2+} solutions were significant ($P = 0.01$). No significant difference was found between responses evoked before and after recovery from high- Ca^{2+} solutions ($P > 0.05$). Elevation of external magnesium from 1.7 to 20 mM ($n=4$) or to 11 mM ($n=1$) had no effect on any of the Ca^{2+} -evoked responses.

B Prolongation of the Ca^{2+} -evoked hyperpolarisation by La^{3+} .

Lanthanum is known to inhibit the Ca^{2+} -ATPase in human red blood cells (Sarkadi et al, 1977). Comparison of Ca^{2+} -evoked responses elicited in high external Ca^{2+} (response B of Fig.4.1) with responses elicited in the presence of 1 mM La^{3+} (response D, Fig.4.1) shows that La^{3+} was more effective in prolonging the duration of the hyperpolarisations. In addition semilogarithmic plots of the percentage fall in input resistance against time corresponding to responses A, B, C, D and E of Fig.4.1 showed that La^{3+} was also more effective than high Ca^{2+} in prolonging both the duration for which the reduction in input resistance remained maximal and the time constant for the recovery of the input resistance (T_R).

In seven experiments (including the one shown in Fig.4.2) the values of $T_{1/2(V)}$ and T_R of responses evoked in the presence of external La^{3+} were 40 ± 29 s (range 25-100 s) and 52 ± 40 s (range 12-115 s) respectively. The values of $T_{1/2(V)}$ before and after La^{3+} treatment were 17 ± 5 s (range 13-26 s) and 16 ± 6 s (range 7-24 s) respectively. The corresponding values of T_R were 10 ± 5 s (range 4-18) and 10 ± 6 s (range 5-21, $n=5$). In two of the above seven experiments, technical problems, i.e. blocking of the Ca^{2+} pipette or spontaneous withdrawal of the recording microelectrode from the egg prevented recovery from the La^{3+} treatment.

C Prolongation of the Ca^{2+} -evoked hyperpolarisation by the flavone quercetin.

The flavone quercetin has been reported to inhibit the Ca^{2+} -ATPase of mouse fibroblasts (Okada, et al, 1982). When quercetin was present in the bathing solution at concentrations in the range 50-500 μM it prolonged the hyperpolarisation evoked by Ca^{2+} injection (Fig. 4.3). Both $T_{1/2(V)}$ and T_R increased without any alteration of the value of the reversal potential for the response (Table 4.1).

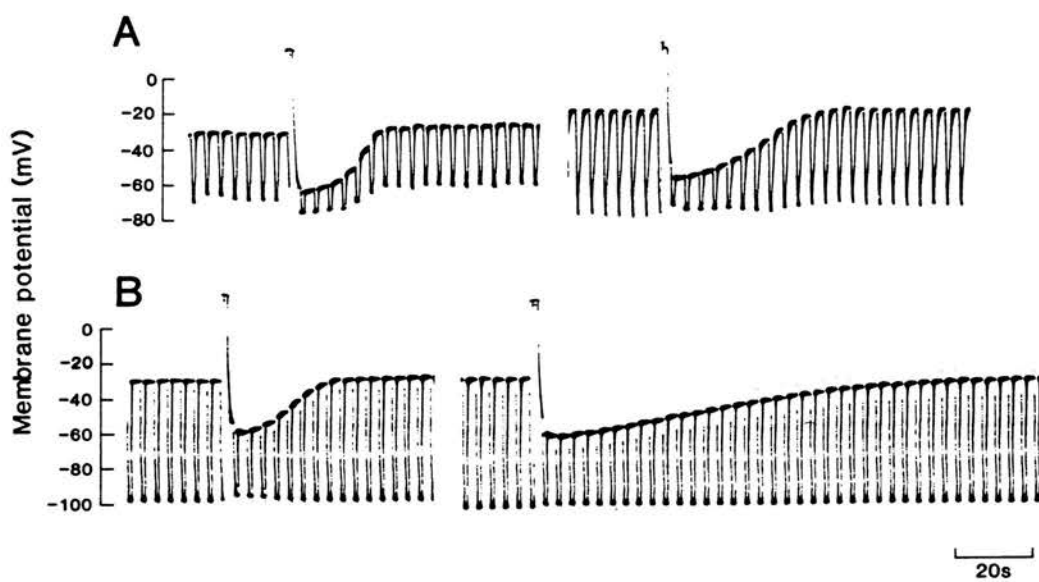


Figure 4.3. Effect of quercetin on the hyperpolarising responses of the egg to calcium-injection. **A** shows responses before (left) and during (right) exposure to 0.1 mM quercetin in the bathing solution. Brief downward deflexions of the membrane potential recordings are electrotonic potentials produced by constant-current pulses (not shown) passed through the recording micro-electrode. **B** shows response of another egg before (left) and during (right) exposure to 0.5 mM quercetin in the bathing solution.

Cell no.	Control			Quercetin			Concn of Quercetin
	ER (mV)	T1/2(v) (s)	TR (s)	ER (mV)	T1/2(v) (s)	TR (s)	(μ M)
1	-91	22	18	-100	45	47	500
2	-76	16	10	-77	23	18	100
3	-71	11	5	-71	27	26	100
4	-81	14	7	-93	28	17	100
5	-72	9	6	-68	60	76	50
6	-75	13	10	-79	20	11	50

Table 4.1 Effects of Quercetin on reversal potential and time course of calcium - evoked hyperpolarisation.

The results obtained with quercetin resembled those with La^{3+} except that the action of quercetin was not truly reversible. Because the quercetin solution contained dimethylsulphoxide (Sigma) it was necessary to examine the effects of dimethylsulphoxide. It produced no change in the time course of the Ca^{2+} -evoked hyperpolarisation.

D Prolongation of the Ca^{2+} -evoked hyperpolarisation by DNP.

DNP acts as an uncoupler of oxidative phosphorylation, removing the H^+ gradient from mitochondria that is necessary for the generation of ATP. As the Ca^{2+} -ATPase requires energy in the form of ATP, the effect of this chemical on the time course of the Ca^{2+} -evoked response was investigated. Bath application of DNP (0.2 - 2 mM) also markedly lengthened the duration of the Ca^{2+} -evoked hyperpolarisation in a reversible manner (Fig. 4.4).

Unlike high Ca^{2+} , La^{3+} or quercetin the effect of DNP was to selectively prolong the period for which the conductance rise was maximal. As shown in Fig. 4.4, the time courses of the return of both potential and resistance from their plateaux to their resting values in responses elicited in the presence of DNP were indistinguishable from those of responses elicited in normal solution. Hence $T_{1/2(V)}$ was chosen as the measure most appropriate in quantifying the effect of DNP. In eight experiments (including the one shown in Fig.4.4) the value of $T_{1/2(V)}$ of responses evoked in normal and in DNP containing solutions was 26 ± 8 and 56 ± 36 s respectively. In five of the eight experiments the effect of DNP was fully reversed whereas in the other three experiments the time course of recovery from DNP treatment could not be examined either because the Ca^{2+} micro-electrode became blocked or the recording micro-electrode dislodged itself from the egg. The value of $T_{1/2(V)}$ for responses obtained after recovery was 21 ± 12 s ($n=5$). Treatment with DNP did not significantly affect the reversal potential. Its values in normal, DNP and normal (recovery) solutions were -71 ± 13 ($n=8$), -76 ± 12 ($n=8$) and -69 ± 11 mV ($n=5$) respectively.

The DNP-induced prolongation of the Ca^{2+} evoked response might be due to a reduction in the intracellular Ca^{2+} buffering capacity of the egg. If this is the case then it would be expected that in the presence of DNP, Ca^{2+} pulses of reduced amplitude would elicit responses of comparable duration to responses evoked by larger Ca^{2+} pulses in the absence of DNP. Fig.4.5 shows the results of one of three separate experiments designed to test this hypothesis. Response A was elicited in normal solution by the injection of Ca^{2+} into an egg. A similar Ca^{2+} injection in the presence of DNP evoked a considerably larger response (B). Response C was elicited in the presence of DNP. In order to get a response of comparable duration in normal solution a larger injection pulse was required. These results were confirmed in two other experiments; they are consistent with the hypothesis that DNP reduced the ability of the egg to buffer effectively the intracellular Ca^{2+} concentration.

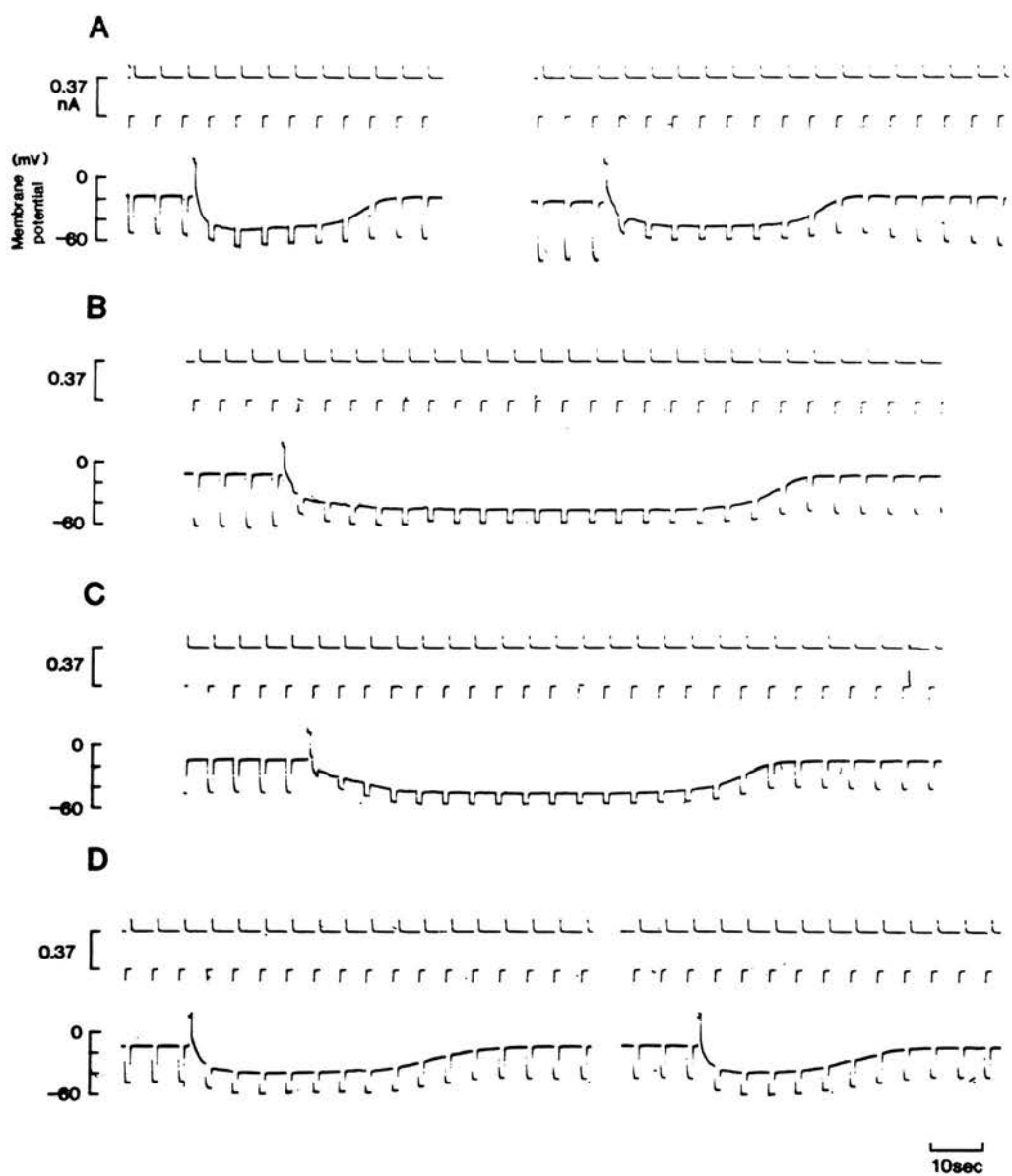


Figure 4.4. The prolonging effect of DNP on the duration of calcium-evoked responses to identical current pulses passed through the calcium micro-pipette. Responses shown in **A** and **D** were obtained in normal solution whereas responses shown in **B** and **C** were obtained in the presence of 0.02 mM DNP. The order in which the responses have been labelled follows the order in which they were obtained. In each example the upper trace shows current and the lower membrane potential.

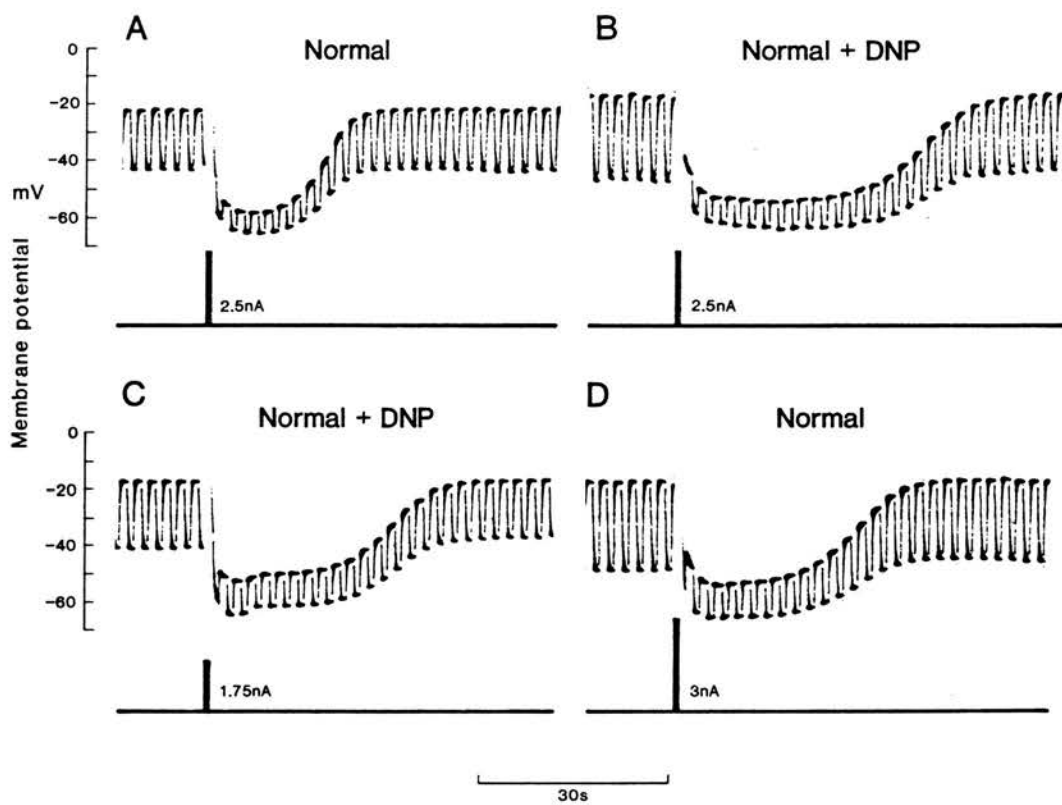


Figure 4.5. Effect of DNP on the relation between the size of the current pulse injected through a calcium micropipette and the duration of the response from an egg. The amplitude of the calcium current pulse is indicated below each membrane potential trace. In all cases the calcium pulse had a duration of 1 s. The brief downward deflexions of the membrane potential are electrotonic potentials evoked by the passage of constant current pulses (not shown) through the recording micro-electrode.

E The enhancing effects of pH_o on hyperpolarisations evoked by Ca^{2+} injection

The results above show that bath application of either La^{3+} , high Ca^{2+} or quercetin which have been reported to inhibit active Ca^{2+} efflux (Dipolo & Beauge, 1982; Okada, Tsuchiya & Yada, 1982) prolong the recovery phase of hyperpolarisations evoked by injections of Ca^{2+} (Georgiou et al, 1987).

High extracellular pH (pH_o) is also reported to inhibit active Ca^{2+} extrusion in squid giant axons (Dipolo and Beauge, 1982). A prolongation of the Ca^{2+} -evoked hyperpolarisation in hamster eggs similar to that discussed above might then be expected if active Ca^{2+} extrusion were inhibited by a rise in pH_o .

To examine this possibility, Ca^{2+} was injected iontophoretically into eggs in bathing solutions of varying pH_o . In five experiments, one of which is illustrated in Fig.4.6, a rise in pH_o from 7.5 to 8.5 caused in all cells but one an increase in the amplitude of the Ca^{2+} -evoked hyperpolarisation. The measured amplitudes were 28, 25, 33, 27 and 47 mV in pH 7.5 and 35, 37, 42, 38 and 43 mV respectively in pH 8.5. In all cells the rise in pH_o caused a substantial reduction in the recovery rate of the membrane potential.

The parameter chosen to measure the duration of the responses in raised pH_o was $T_{(V)}$; this is the duration of the response at half its maximum amplitude, measured from the peak of the response (illustrated in Fig. 4.2). $T_{(V)}$ increased approximately fourfold from 4.3, 4.2, 6.6, 5.0 and 4.2 s to 18.4, 21.0, 16.0, 19.0 and 27 s respectively. In three of the above experiments it was possible to obtain values for amplitude and $T_{(V)}$ after recovery in normal solution; the values of amplitudes were 33, 33 and 51 mV and the values of $T_{(V)}$ were 10.6, 5.6, and 4.5 s respectively. In the other two experiments Ca^{2+} micropipette blockage prevented the induction of responses in normal (recovery) solution. A similar reversible prolongation was observed at the higher temperature of 35-37 °C (n=2).

F The effect of Na^+ removal on the properties of Ca^{2+} -evoked hyperpolarisations

Igusa and Miyazaki (1983) reported that the duration of the hyperpolarisation evoked by intracellular injection of Ca^{2+} was markedly prolonged (i.e. by 3-4 times) in solutions where external Na^+ was replaced by either Li^+ or $Tris^+$. They tentatively attributed the increase in duration to the inhibition of a Na^+ - Ca^{2+} exchange mechanism.

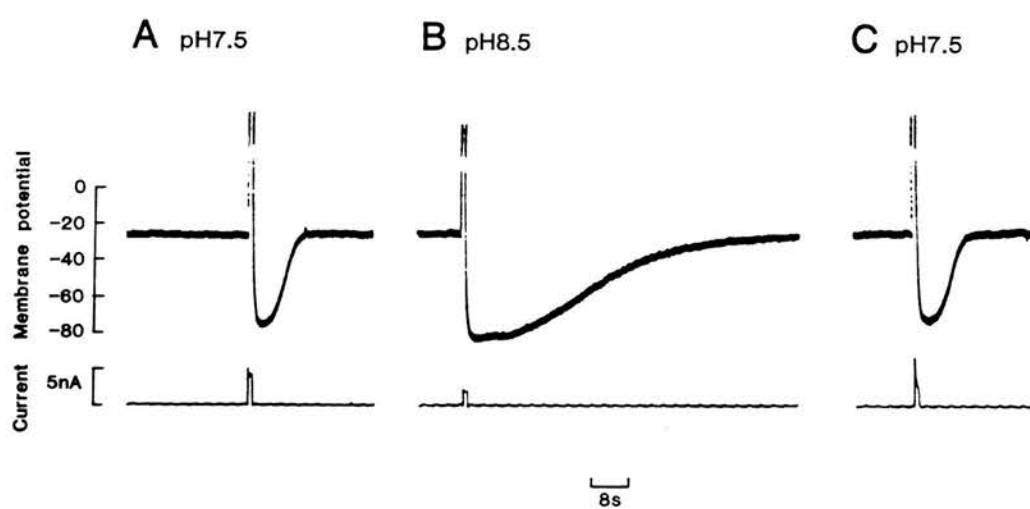
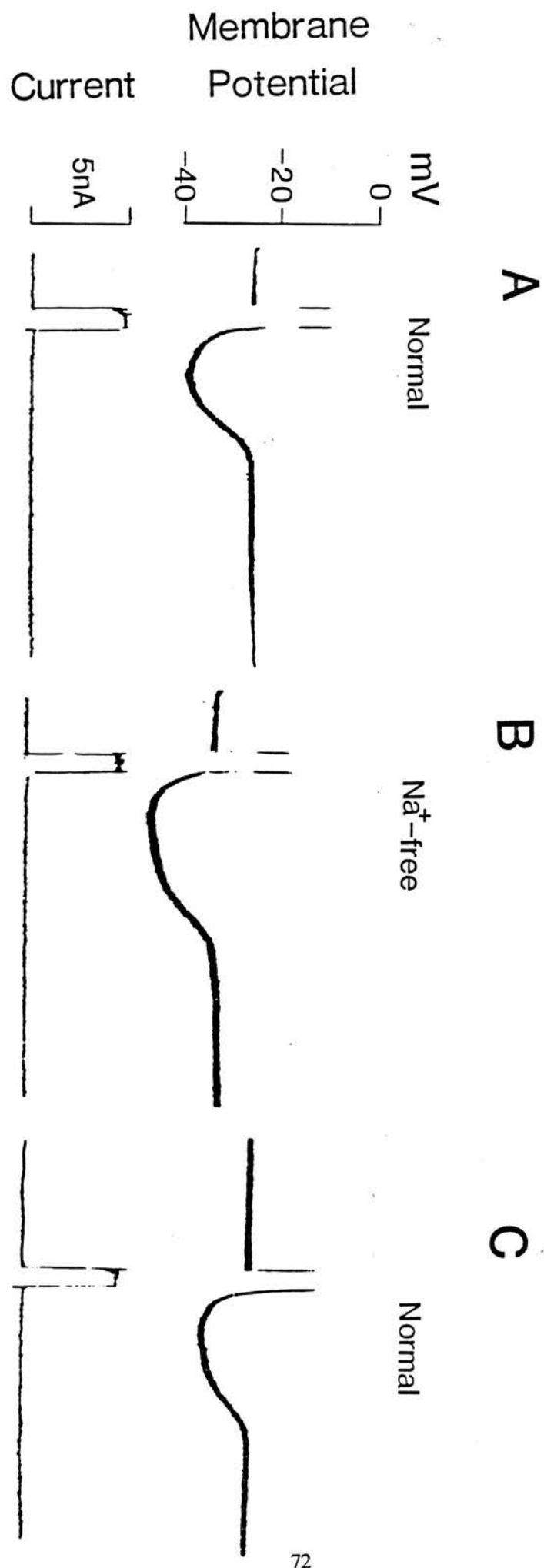


Figure 4.6. Prolongation of the response induced by iontophoretic injection of calcium into an egg bathed in a solution of high pH. Note that the injected current in **B** is smaller than in **A** and **C**. Upper traces show membrane potentials and lower traces show the current pulse injected into the egg through the calcium micropipette.



5s

Figure 4.7. Reversible prolongation of the calcium-induced hyperpolarisation by sodium-free solution at 35°C. **A** and **C** are hyperpolarisations evoked in normal solution. **B** is a response evoked in a solution where sodium has been replaced by N-methyl-D-glucamine. Injected currents are shown in lower traces, membrane potentials in the upper traces.

The prolongation of the Ca^{2+} -evoked hyperpolarisation caused by raised pH_o might have been due to an inhibition of this Na^+ - Ca^{2+} exchange, so the operation of such an exchange system was investigated. Preliminary experiments were performed using Li^+ as a partial replacement for Na^+ (1/6 Na^+ , 5/6 Li^+) as complete replacement of Na^+ by Li^+ appeared to cause the cell irreparable damage (irreversible depolarisation and loss of input resistance). In three experiments at 35-37 °C ($[\text{Ca}^{2+}]_o=1.7$), $T_{(V)}$ was prolonged by a factor of 1.3 from a value of 1.4 ± 0.3 s in normal solution to a value of 1.9 ± 0.4 s in Li^+ solution.

At the same temperature but with $[\text{Ca}^{2+}]_o = 4$ mM, $T_{(V)}$ was prolonged in two eggs from 1.3 s in normal solution to 2.5 and 2.0 s in lithium solution. In these experiments, substitution of Na^+ by Li^+ resulted in a depolarisation of about 10 mV and a rise in conductance. These changes indicate that Li^+ is not a good substitute for Na^+ and therefore N-methyl-D-glucamine (NMDG) was chosen because it does not cause any depolarisation (Nachshen, Sanchez-Armass & Weinstein, 1987).

Substitution of Na^+ by NMDG usually produced a membrane hyperpolarisation, for example, from -17, -19, -24 and -16 in control solution to values of -34, -33, -34 and -43 mV in Na^+ -free solution for four eggs. In experiments on four eggs at 35-37 °C where Na^+ was replaced by NMDG, the value of $T_{(V)}$ was prolonged from 1.9 ± 0.5 s ($n=10$) in control solutions to 3.6 ± 0.9 s ($n=10$) in Na^+ -free solution. An example of the prolongation of the Ca^{2+} -induced hyperpolarisation by Na^+ removal at 35 °C is illustrated in Fig.4.7. A similar prolongation of $T_{(V)}$ was observed in additional experiments performed at room temperature (21-25 °C). Here, $T_{(V)}$ increased from 9.9 ± 3.0 s ($n=9$) in control solution to 17.5 ± 7.2 s ($n=9$) in Na^+ -free solution.

Removal of Na^+ might have affected other systems such as a Na^+ - H^+ exchanger or a Na^+ - K^+ pump. Also raising $[\text{Ca}^{2+}]_o$ from 4 to 10 mM is known to block the Na^+ - H^+ exchanger in frog muscle (Putnam & Roos, 1986). Application of inhibitors of these systems such as 1 mM amiloride (Putnam, Roos & Wilding, 1986), and 1mM ouabain did not produce any changes in the electrical properties of the eggs.

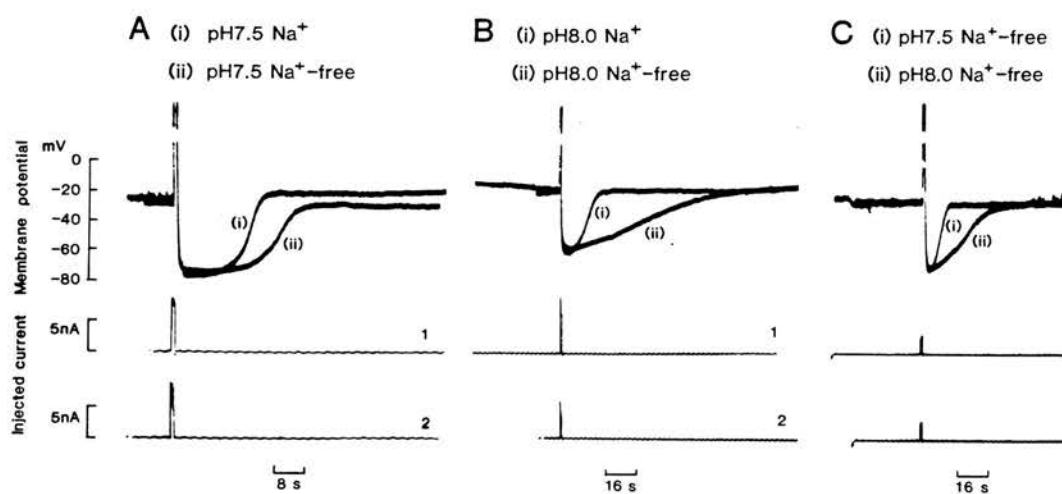


Figure 4.8. Prolongation of the responses evoked by calcium injection in sodium-free solutions.

Responses from the same cell are shown in superimposed traces. Injected currents are shown in lower traces. **A** in the presence (trace (i) and current 1) and in the absence (trace (ii) and current 2) of sodium in bathing solutions at pH 7.5. **B**, in the presence of (trace (i) and current 1) and in the absence (trace (ii) and current 2) of external sodium at pH 8.0. **C**, at pH 7.5 (trace (i) and current 1) and at pH 8.0 (trace (ii) and current 2) in the absence of external sodium.

G The effect of pH_o on the Ca^{2+} -evoked hyperpolarisation, in the absence of Na^+ .

The results above suggested that a Na^+ - Ca^{2+} exchange was present in the hamster egg. Dipolo & Beauge (1982) reported that raising the extracellular pH_o of squid axons inhibited a Ca^{2+} pump. The effect of raising pH_o and Na^+ removal on the Ca^{2+} -evoked hyperpolarisation was therefore examined. To compare the effects on the time course of the hyperpolarisations, responses recorded in different solutions were superimposed (Fig.4.8). The duration of the hyperpolarising response to Ca^{2+} injection at pH_o 7.5 was increased in Na^+ -free solution compared to normal solution. This moderate increase consisted of a prolongation of the plateau phase and a slowing of the recovery phase; traces of responses evoked in different solutions were superimposed for an easy comparison (Fig. 4.8A, i & ii). At higher pH_o , however, the response was greatly prolonged on removal of sodium, mainly due to the slowing of the recovery phase (Fig.4.8B, i & ii). There was little change in the time course of the responses evoked in the presence of external Na^+ at pH_o 7.5 and 8.0 (Figs. 4.8A i & 4.8B i); the recovery phase was slightly slowed at pH_o 8.0 in one experiment out of three. The effects of raising pH_o while the putative Na^+ - Ca^{2+} exchange system was suppressed was then examined. In eggs bathed in Na^+ -free solution, raising the pH_o from 7.5 to 8.0 produced a marked slowing of the recovery phase (Fig. 4.8C, i & ii). This suggests that when the pH_o is raised in the presence of Na^+ , an inhibitory effect of the high pH_o is being masked, possibly by the compensatory action of a Na^+ - Ca^{2+} exchange system extruding Ca^{2+} .

H Transient hyperpolarisations induced by Na^+ -free solution

If a Na^+ - Ca^{2+} exchange system were present in the egg, it might be possible to reverse the direction of the exchange by the removal of extracellular Na^+ and raising $[\text{Ca}^{2+}]_o$ so as to cause a rise in $[\text{Ca}^{2+}]_i$. This effect would be enhanced by the increase of the electrochemical gradient for Ca^{2+} , possibly increasing Ca^{2+} influx through non-voltage gated Ca^{2+} channels (the involvement of Ca^{2+} channels in the electrical activity of the hamster egg is discussed in the next Section). These combined effects would lead to the activation of the Ca^{2+} -mediated K^+ conductance.

In three experiments, complete substitution of Na^+ with NMDG in the presence of 20 mM Ca^{2+}_o resulted in the generation of one or two transient hyperpolarisations as illustrated in Fig. 4.9. The responses were accompanied by a large rise in membrane input conductance and had a reversal potential of -74.8 ± 5.8 mV ($n=3$), compatible with E_K .

pH7.5 Na^+ -free 20mM Ca^{2+}

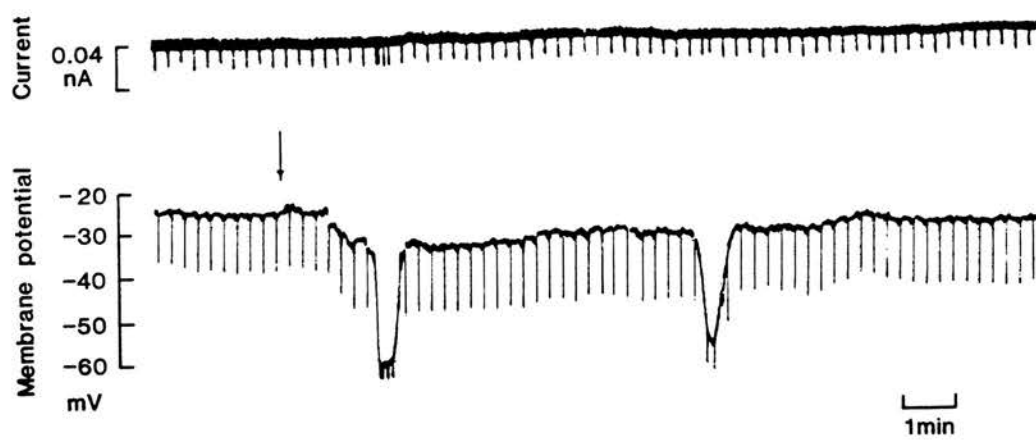


Figure 4.9. Transient hyperpolarisations obtained in Na^+ -free, 20 mM Ca^{2+} solution. Upper trace shows the current, lower trace shows the membrane potential.

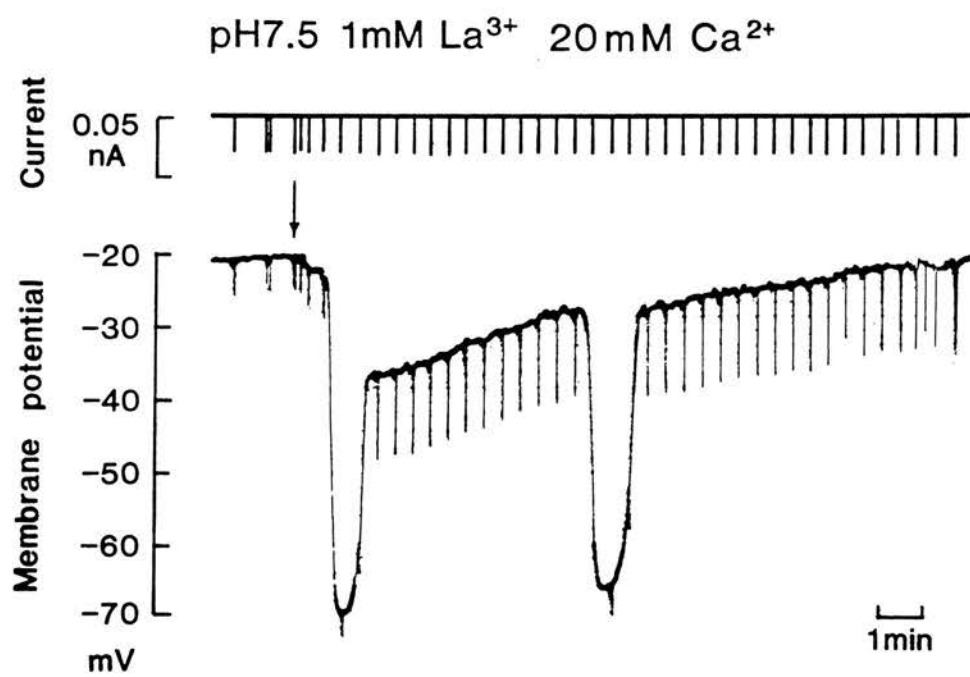


Fig.4.10. Transient hyperpolarisations obtained in 1 mM La^{3+} , 20 mM Ca^{2+} solution. Upper trace shows current, lower trace shows the membrane potential.

I Transient hyperpolarisations induced by La^{3+}

The recovery phase of the Ca^{2+} -evoked hyperpolarisation is considerably prolonged in the presence of external La^{3+} (Georgiou et al, 1987a), a result consistent with reports that La^{3+} inhibits active Ca^{2+} efflux (Sarkadi, et al., 1977; Baker & Dipolo, 1984).

As described earlier, the effect of La^{3+} on the passive electrical properties of hamster eggs (Section 3) was to produce a steady hyperpolarisation of about 20 mV. As this hyperpolarisation was accompanied by approximately a twofold rise in input resistance rather than a fall, as would be expected if channels were opened, it was attributed to an increase in the micro-electrode seal resistance. Those experiments were carried out in solutions where $[\text{Ca}^{2+}]_o$ was 4 mM. Since high $[\text{Ca}^{2+}]_o$ is known to inhibit Ca^{2+} efflux (Dipolo & Beauge, 1983), the effect of La^{3+} in the presence of high Ca^{2+} (20 mM) was examined, to see if the two ions would act synergistically, leading to a hyperpolarisation and rise in membrane conductance.

The outcome of one representative experiment out of three is illustrated in Fig. 4.10, showing that the combined effect of La^{3+} and high Ca^{2+} was the induction of one or two transient hyperpolarisations similar to those induced by high Ca^{2+} in Na^+ -free solution. Again the input conductance rose approximately fourfold at the peak of the responses, indicating channel opening, and reversal potentials of -84.4 ± 3.6 mV were observed.

Discussion.

The micro-injection of Ca^{2+} ions into hamster eggs produces a transient hyperpolarisation resulting from the opening of potassium channels (Igusa and Miyazaki, 1983; Georgiou et al, 1983). Cytosolic Ca^{2+} concentrations of 0.7 to 2 μM are required to produce these hyperpolarisations (Igusa and Miyazaki, 1986). The time course of the recovery of the Ca^{2+} -evoked responses was examined in eggs bathed in solutions likely to interfere with Ca^{2+} extrusion.

Effects of La^{3+} , quercetin and high Ca^{2+} concentration.

La^{3+} and quercetin, which are known to reduce the activity of membrane Ca^{2+} -ATPases (Sarkadi et al.1977; Okada et al.1982), greatly prolonged the recovery of both the membrane potential and input resistance of Ca^{2+} -evoked responses (figs. 4.1 & 4.3). La^{3+} does not pass through the egg plasma membrane (House & Bland, 1983), suggesting an external site of action. These findings suggest that the egg's plasma membrane has a Ca^{2+} pump which extrudes Ca^{2+} from the cytosol. Consistent with this view is the prolongation of the Ca^{2+} -evoked

hyperpolarisation seen on raising the extracellular Ca^{2+} concentration (fig.4.1B); Dipolo and Beauge (1982) report that high extracellular Ca^{2+} inhibits a Ca^{2+} -ATPase in squid axons.

The possibility that La^{3+} may block a Na^{+} - Ca^{2+} exchanger, as it does in cardiac cells (Kimura, Noma and Irisawa, 1986) has not been eliminated. Quercetin has not been reported to inhibit the Na^{+} - Ca^{2+} exchanger in other systems, but it is known to inhibit the Na^{+} - K^{+} ATPase (Kuriki & Racker, 1976) and the Ca^{2+} -ATPase of sarcoplasmic reticulum (Fewtrell & Gomperts, 1977).

The slowing of the recovery phase of the Ca^{2+} -evoked hyperpolarization produced by La^{3+} , quercetin or extracellular Ca^{2+} was not associated with a prominent change in the response's reversal potential. In the absence of those substances, responses to Ca^{2+} injection were sometimes equally prolonged. However, in such cases a marked decrease of reversal potential was observed. As similar long responses were evoked by injection of other ions that do not activate the Ca^{2+} -activated K^{+} channel in the absence of sufficient Ca^{2+} , for example Mg^{2+} (Fig 3.4, Section 3), it seems likely that the large depolarisation caused by the iontophoretic pulse sometimes leads to membrane breakdown.

The effect of intense voltages on biological membranes was reported first by Hodgkin (1947), who observed that non-medullated axons suffered a gradual loss in resistance when subjected to excessive polarisations. Del Castillo & Katz (1954) found a similar phenomenon for the nodes of Ranvier of frog sciatic nerves. They observed that 'anodic break-down' occurred when the node was polarised to voltages lying in the range -150 to -400 mV. The voltages (+150 mV) associated with iontophoretic Ca^{2+} injection might also occasionally produce a transient non-selective increase in ion permeability of the egg's plasma membrane.

Effects of DNP.

Calcium-evoked responses were also prolonged in the presence of DNP. As in the case of La^{3+} or quercetin this effect was produced without a change in the reversal potential. However, the DNP-induced prolongation was achieved by a selective extension of the plateau phase and not the recovery phase. Georgiou et al (1983) had shown that responses to large pulses of Ca^{2+} had long plateau phases whereas small pulses caused transient responses without plateaux.

Thus, a possible explanation for the effect of DNP is that it reduces the ability of the egg to buffer its cytosolic Ca^{2+} , possibly by inhibiting active Ca^{2+} uptake into intracellular stores. In that case, the injected Ca^{2+} pulse will produce a larger and more prolonged rise in the cytosolic Ca^{2+} concentration than normal for that pulse. Such an effect would selectively lengthen the plateau phase of the response.

Effects of high pH_o .

Alkaline pH_o is known to reversibly inhibit Ca^{2+} efflux via the uncoupled Ca^{2+} pump in squid giant axons (Dipolo & Beauge, 1982). In the hamster egg, the recovery phase of the Ca^{2+} -evoked response is reversibly slowed by high pH_o , consistent with the interpretation that the inhibition of a Ca^{2+} pump occurs. This leads to a rise in intracellular free Ca^{2+} , activating a Ca^{2+} -activated K^+ conductance.

Involvement of the Na^+ - Ca^{2+} exchange.

Although an increase in duration of the Ca^{2+} -evoked response in sodium-free solution was seen, it was not as large as the 3-4 times increase observed by Miyazaki & Igusa (1983). The buffers they used ($NaHCO_3$ or Tris), however, were possibly not sufficient to prevent a slight drift in pH_o toward the alkaline. The combination of alkaline pH_o and Na^+ -free solution would be potent in prolonging a Ca^{2+} -evoked response, as in Fig. 4.8B. The results obtained at pH 7.5 did suggest that a Na^+ - Ca^{2+} exchange was present in the hamster egg.

It was then necessary to determine if raising the pH_o had any effect on this extrusion system. Hodgkin and Nunn (1987) have reported that high pH_o facilitates the Na^+ - Ca^{2+} exchange in salamander rods. The effect of high pH_o on the Na^+ - Ca^{2+} exchange could be examined by the experiments shown in Fig. 4.8B. At normal pH 7.5, the recovery phase of the Ca^{2+} evoked response was slightly slowed and prolonged in duration by removal of Na^+ from the bathing solution (Fig. 4.8A). In contrast, at high pH_o , the recovery phase was greatly prolonged by Na^+ removal (Fig. 8B). These results were obtained by removing Na^+ from the external solution at a given pH.

The observed differences in responses can be attributed to the activity of the Na^+ - Ca^{2+} exchange system. If high pH_o had no effect on the Na^+ - Ca^{2+} exchange, prolongation of the response at high pH_o (Fig. 4.8B) should be similar to the prolongation at normal pH (Fig. 4.8A). Thus it is suggested that the Na^+ - Ca^{2+} exchange was facilitated at high pH and it made the difference in the recovery phase much larger at high pH (Fig. 4.8B).

Further evidence to support the possibility of facilitation of the Na^+ - Ca^{2+} exchange at high pH_o is that there was little difference in the recovery phase of the response to Ca^{2+} injection between 7.5 and 8.0 when Na^+ was present. It is considered that the inhibition of the Ca^{2+} pump activity was counterbalanced by facilitation of the Na^+ - Ca^{2+} exchange at high pH_o . Fig. 4.8C shows the effect of high pH_o on the Ca^{2+} pump mechanism after removal of the Na^+ - Ca^{2+} exchange using Na^+ -free solution; the recovery phase of the response was greatly prolonged by high pH_o , indicating inhibition of the Ca^{2+} pump.

Inhibition of the ATP-dependent Ca^{2+} pump mechanism in squid axon found at high pH_o is significantly potentiated by external Ca^{2+} ions (Dipolo & Beauge, 1982). This effect, originally found and studied in the sarcoplasmic reticulum Ca^{2+} pump (De Meiss & Martins, 1980) has been related to facilitation of the reversal cycle of the Ca^{2+} pump (ATP synthesis).

It is concluded that both the Na^+ - Ca^{2+} exchange and the Ca^{2+} pump are contributing to the recovery phase of the hyperpolarising response, and that the inhibition of the Ca^{2+} pump is greater than the facilitation of the Na^+ - Ca^{2+} exchange at a raised pH. Thus high pH_o probably causes a rise in intracellular Ca^{2+} , and this may be amplified by processes suggested by findings discussed in Sections 5 and 6.

Further evidence suggesting the involvement of Ca^{2+} extrusion systems comes from the experiments with high Ca^{2+} in the presence of either La^{3+} (Fig.4.10) or Na^+ -free solution (Fig.4.9), at pH 7.5. Under both conditions (in the absence of Ca^{2+} injections), the Ca^{2+} efflux was reduced and the Ca^{2+} influx augmented, resulting in hyperpolarisations due to Ca^{2+} -mediated K^+ channels. The latter (Na^+ -free) results, are interesting in the light of cardio-vascular research; it is common practice to reverse the Na^+ - Ca^{2+} exchanger in heart cells in order to produce a rise in $[\text{Ca}^{2+}]_i$, by removing extracellular Na^+ (Reuter and Seitz, 1968).

SECTION 5 Investigation of a high pH_o -induced hyperpolarisation.

The results presented in this section form an investigation of the effects of raising pH_o on the electrical properties of the hamster egg. The effects of pH_o on mammalian eggs are of general interest because of the influence of pH on fertilisation. The pH of the fluid in the reproductive tracts of several mammalian species lies in the range 7.10 - 8.04 (Hall, 1936; Blandau, Jensen & Rummery, 1958). In particular, Vishwakarma (1962) found that the pH of the fluid in the ligated oviducts of rabbits was about 7.9.

It is possible that the mild alkalinity of the oviducal fluid favours sperm penetration. This idea is supported by the observation that optimal rates (93-99 %) of fertilisation in vitro of hamster eggs occurred in the range 7.0 - 8.5 (Miyamoto, Toyoda & Chang, 1974). Moreover, accompanying fertilisation is a marked rise in intracellular pH in the eggs of sea urchin (Shen & Stenhardt, 1978), sand dollar (Hamaguchi, 1982) and frog (Webb & Nuccitelli, 1981). This change in intracellular pH probably has an important regulatory role in a number of processes, such as DNA and protein synthesis occurring in fertilised eggs (Busa & Nuccitelli, 1984; Swann and Whitaker, 1987).

The effects of raising the pH of the solution bathing the hamster egg are discussed in the light of results obtained by workers in other preparations.

Results

A The effect of changes of pH_o on membrane potential and input resistance.

When the pH of the normal solution bathing an egg at room temperature (21-24 °C) was raised from 7.5 to 8.0 a transient hyperpolarisation lasting about 20 s was recorded occasionally. At pH_o values above 8.0, a transient hyperpolarisation frequently was observed and it was followed by a further maintained hyperpolarisation. An example of this is illustrated in Fig.5.1 A1. A burst of action potentials sometimes accompanied the recovery phase of the transient hyperpolarisation. These are due to Ca^{2+} influx through voltage-gated Ca^{2+} channels shown to exist in these cells, after removal of inactivation (Miyazaki and Igusa, 1982b). An example is illustrated at a faster time-base in Fig.5.1 A2.

The response to high pH_o could be reversed to a depolarisation in eggs with membrane potentials shifted to more negative values than -100 mV (Fig. 5.1 B). The time course and amplitude of the response to raised pH_o were not altered significantly by performing experiments at 35-37 °C (n=4). Lowering the pH to 6.5 or 5.0 did not produce a hyperpolarisation in the egg. Instead, a depolarisation of about 10 mV was observed, associated with a small reduction in input resistance.

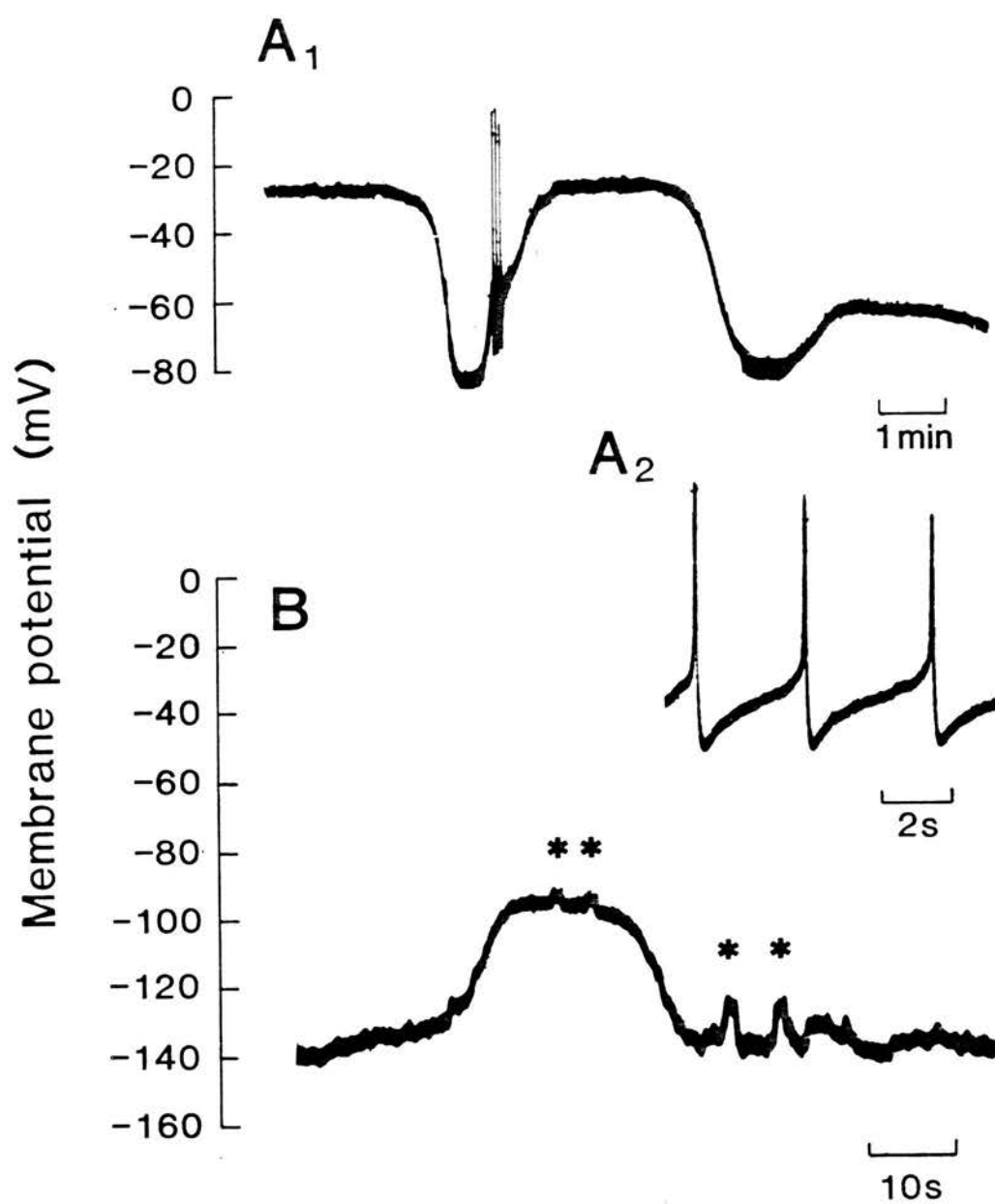


Figure 5.1. Effects of raising pH of a solution bathing a hamster egg above 8.0. **A**, transient, followed by a steady membrane hyperpolarization evoked by raising the pH_o to 9.0 (A1). Action potentials were seen on the recovery phase of the transient hyperpolarisation. These are shown at a faster time base in A2. An under-shoot was apparent at the end of each action potential, probably due to activation of calcium-activated potassium channels. **B**, the response was reversed to a depolarisation by applying pH 8.0 solution to an egg current-clamped to a hyperpolarised potential. Asterisks indicate electrotonic potentials used for monitoring the membrane resistance change..

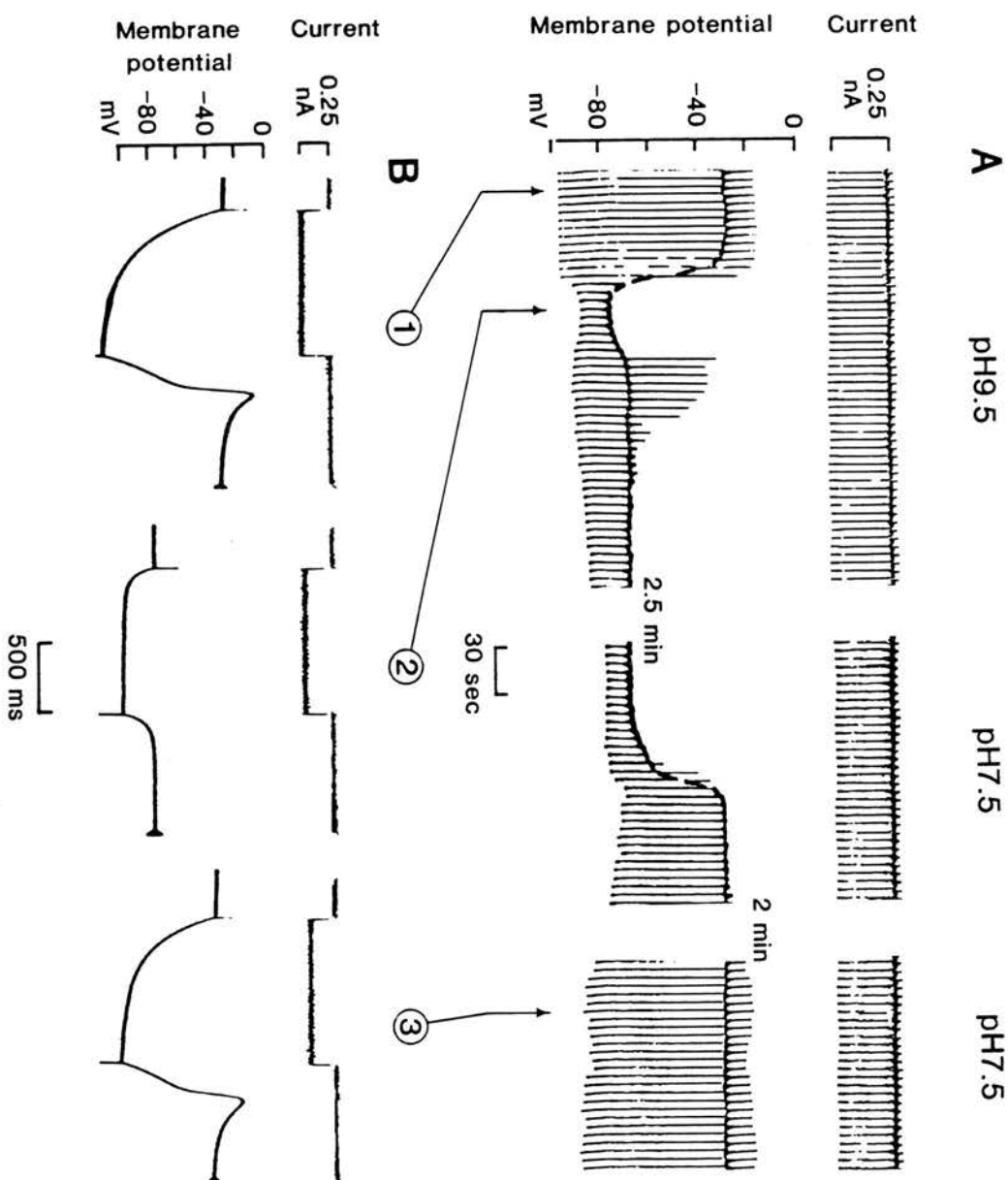


Figure 5.2. Reversible effect of high pH_o (9.5) on the membrane potential and resistance of a hamster egg in solution containing 4 mM calcium. **A**, pen traces of current pulses (upper) and membrane potential (lower). **B**, corresponding oscilloscope pictures at a faster time-base, taken at the time indicated by arrows 1, 2 and 3.

Most responses were recorded at pH 9.5 because at that pH a large steady hyperpolarisation of about 40 mV was usually observed. Eight cells out of a group of twenty-eight responded with a small hyperpolarisation (<10 mV). These are referred to as 'poorly-responding' cells. The hyperpolarisation of responding cells was associated with a marked reduction in input resistance. A representative example of one experiment shows that the hyperpolarisation induced by pH_o 9.5 (Fig.5.2 A) was accompanied by a large fall in resistance from 310 to 90 MΩ at the peak of the response (Fig. 5.2 B. compare electrotonic potentials at 1 and 2). An almost full recovery of potential and resistance was achieved by superfusing the egg with normal solution for 10 min. (Fig. 5.2 A).

Table 5.1 gives the mean values of membrane potentials (E_M) and input resistances (R_{in}) measured before, at the peak of and after recovery from the hyperpolarisation due to high pH_o at 4 mM Ca²⁺ in ten representative experiments. Mean estimates of the reversal potential are also given. The mean membrane conductance increased by 8.9 ± 3.2 nS during the response to high pH_o. The conductance rise due to pH_o application was calculated by subtracting the resting conductance of the egg from the conductance of the egg during the hyperpolarisation. These conductances were measured from the electrotonic potentials before and during high pH application.

In another series of experiments (n=15) made at a [Ca²⁺]_o of 20 mM, elevation of pH_o from 7.5 to 9.5 always elicited a large steady hyperpolarisation. In many cases, the steady hyperpolarisation was preceded by one or two transient hyperpolarisations.

In some eggs a rise of pH_o from 7.5 to 8.0 was sufficient to evoke a transient hyperpolarisation. Mean values of potential and resistance measured before, during and after hyperpolarising responses obtained in the presence of 20 mM Ca²⁺ are given in Table 5.1; the mean values of E_R are also given. The mean membrane conductance increased by 6.8 ± 3.4 nS during the response to high pH_o.

B Rapid application of high-pH solution

The perfusion system used in these experiments was driven by a pump and had a lag time of about 2-3 min before exchange of the bathing solution was completed. This may be due to the large volume of the chamber. In order to examine the speed of onset of the hyperpolarisation, a high-pH solution was applied from a broken-tipped pipette placed close to the egg (Fig.5.3 A).

A hyperpolarisation could be evoked within 10 s of bringing the broken-tipped pipette into close proximity to the egg (Fig.5.3B). The membrane potential recovered fully when the pipette was removed. Such transient hyperpolarisations could be evoked repeatedly in eggs by this method

(Fig. 5.3B & C). Occasionally a burst of action potentials accompanied the recovery phase of the hyperpolarisation (Fig.5.3C).

The mean value of the latency of the pH_o -induced hyperpolarisation elicited by rapid application of high-pH solution was 20.8 ± 11.4 s (n=6). In these experiments, the shortest latency was 6 s.

	Resting		Peak		Recovery		E _R
	EM(mV)	R _{in} (MΩ)	EM	R _{in}	EM	R _{in}	
4mM Ca ²⁺	-27±7	330±130	-65±9	84±23	-29±6	320±55	-80±7
pH 9.5	(n=10)	(n=10)	(n=10)	(n=10)	(n=7)	(n=7)	(n=10)
20mM Ca ²⁺	-30±4	400±84	-73±5	110±33	-32±13	300±130	-87
pH 9.5	(n=15)	(n=15)	(n=15)	(n=15)	(n=14)	(n=14)	(n=15)

Table 5.1. Effects of high pH_o on membrane potential and input resistance. E_M, membrane potential; R_{in}, input resistance; E_R, estimated reversal potential (see Section 2). Values are given in the form mean ± S.D.; numbers of cells are given in parentheses.

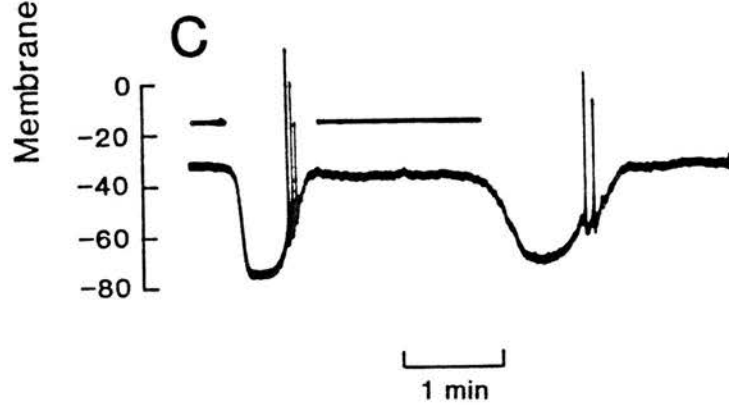
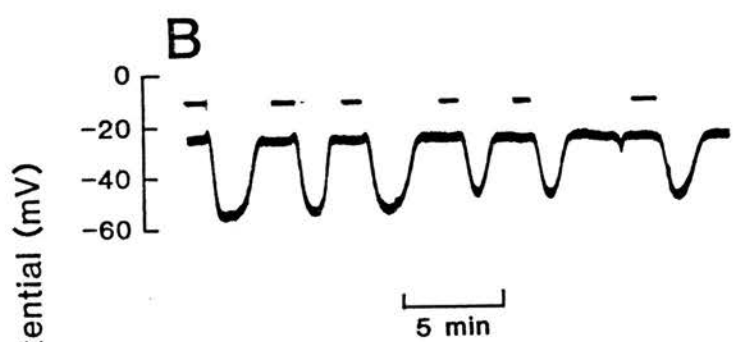
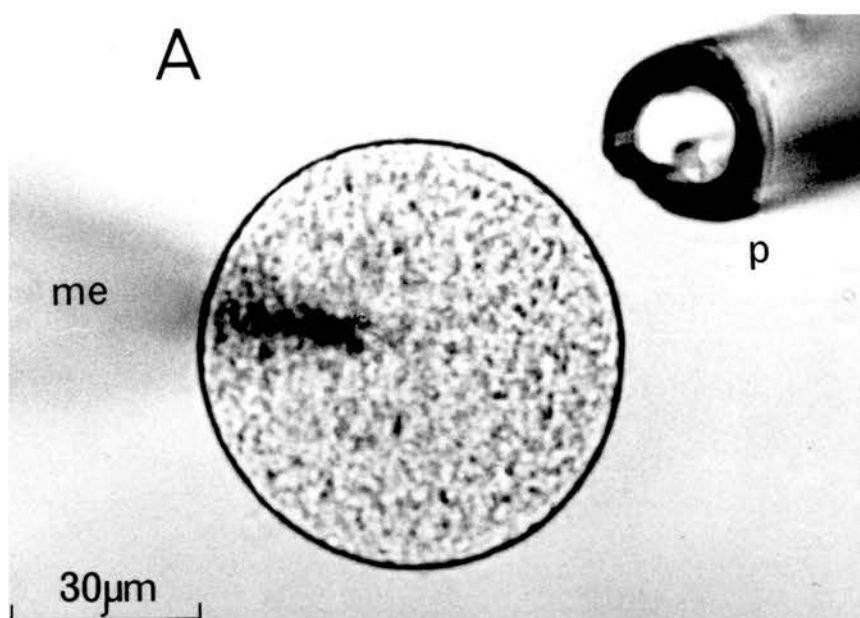


Fig. 5.3. Rapid application of high pH solution to an egg. A, photograph showing the method. Membrane potential was monitored by micro-electrode (m.e.). High pHo (9.5) was applied to the egg from the broken-tip pipette (p.). The pipette was withdrawn as soon as the membrane started to hyperpolarise. The pH of the bathing solution was 7.5. B, the membrane hyperpolarised when the broken-tip pipette was brought close to the egg and recovered on removal of the pipette. Applications are shown by bars. C, another example of the membrane potential change induced by the same method. Action potentials were again seen on the recovery phase of the responses.

C Ionic basis of the hyperpolarisation.

The estimated mean values of E_R (Section 2) given in Table 5.1 are compatible with the equilibrium potential of either K^+ or Cl^- . They are also in good agreement with the reversal potentials obtained from transient hyperpolarisations in fertilised eggs (Miyazaki & Igusa, 1981a) and hyperpolarisations evoked by Ca^{2+} injection (Igusa and Miyazaki, 1983; Georgiou et al, 1987a).

To examine whether K^+ or Cl^- underlies the ionic basis for the response, ionic substitution experiments were performed. In three experiments substitution of external chloride with the impermeant anion methane sulphonate (Sharp & Thomas, 1981) had no effect on either the amplitude of the pH_o -induced hyperpolarisation or the reversal potential of this response. However, the membrane potential at pH_o 9.5 changed linearly with $\log [K^+]_o$ between 5 and 100 mM (Fig.5.4). The mean value of the slope obtained from four such experiments was 43 ± 2 mV for a tenfold change in $[K^+]_o$.

The dependence of the hyperpolarisation on $[K^+]_o$ and the value of the reversal potential indicate that a rise in the membrane K^+ conductance is evoked by high pH_o .

Further evidence that the hyperpolarisation was due to an increase in membrane K^+ conductance came from three experiments where the effect of the K^+ channel blocker tetraethylammonium (TEA) was investigated. External application of 20 mM TEA caused a reduction in the amplitude of the pH_o -induced hyperpolarisation concomitant with a fall in input conductance. The amplitudes of the hyperpolarisations in the presence of 20 mM TEA were 50, 73 and 72 % of the values in the absence of TEA. In one such experiment, TEA was applied in increasing concentration (5, 10 and 20 mM) in a pH 9.5 solution and step-wise reductions in the amplitude of the pH_o -induced hyperpolarisation observed.(Fig.5.5). Recovery of the response could be obtained by washing the egg with normal solution at pH 9.5.

D Ca^{2+} requirement for the initiation and maintenance of the response

If the primary effect of high pH_o were to release Ca^{2+} from intracellular stores, perhaps as a consequence of a rise in intracellular pH, then it might be expected that a hyperpolarisation would be initiated in the absence of external Ca^{2+} .

In three experiments where Ca^{2+} was replaced with Mg^{2+} (see Section 2) a rise in pH_o from 7.5 to 9.5 never elicited a hyperpolarisation. However, when $[Ca^{2+}]_o$ was raised to a sufficient level a hyperpolarising response to high pH_o was evoked (Fig.5.6).

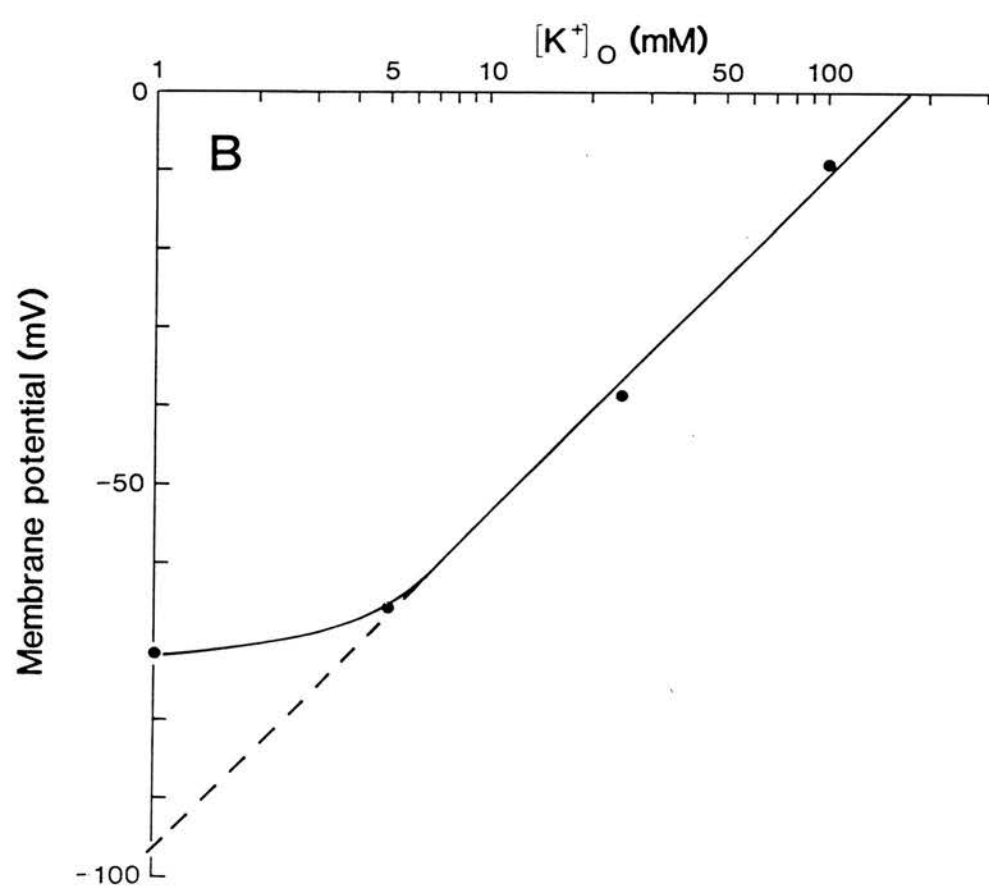
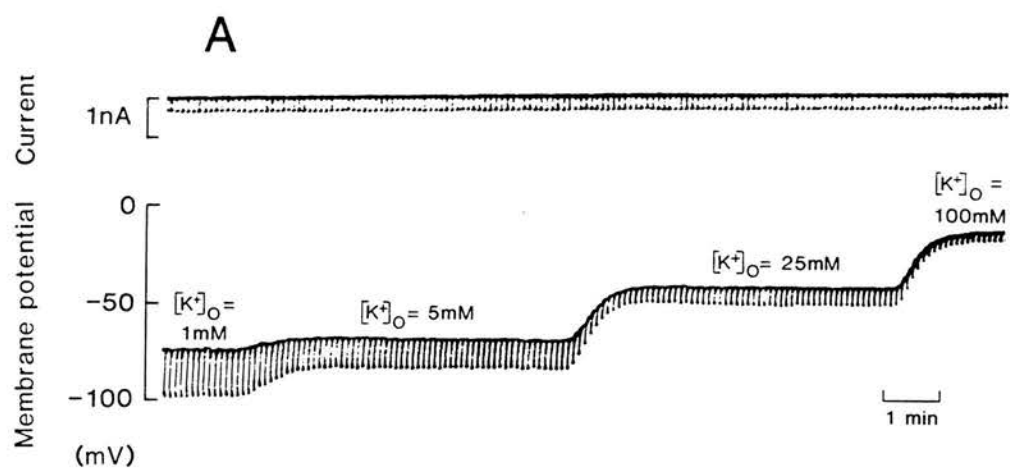


Figure 5.4. The dependence on $[K^+]_o$ of the steady membrane potential at pH 9.5. **A**, pen traces of current pulses (upper) and membrane potential (lower). **B**, membrane potential plotted against $\log [K^+]_o$. The linear part of the relation was fitted by regression analysis and had a slope of 43 mV for a 10-fold change in $[K^+]_o$.

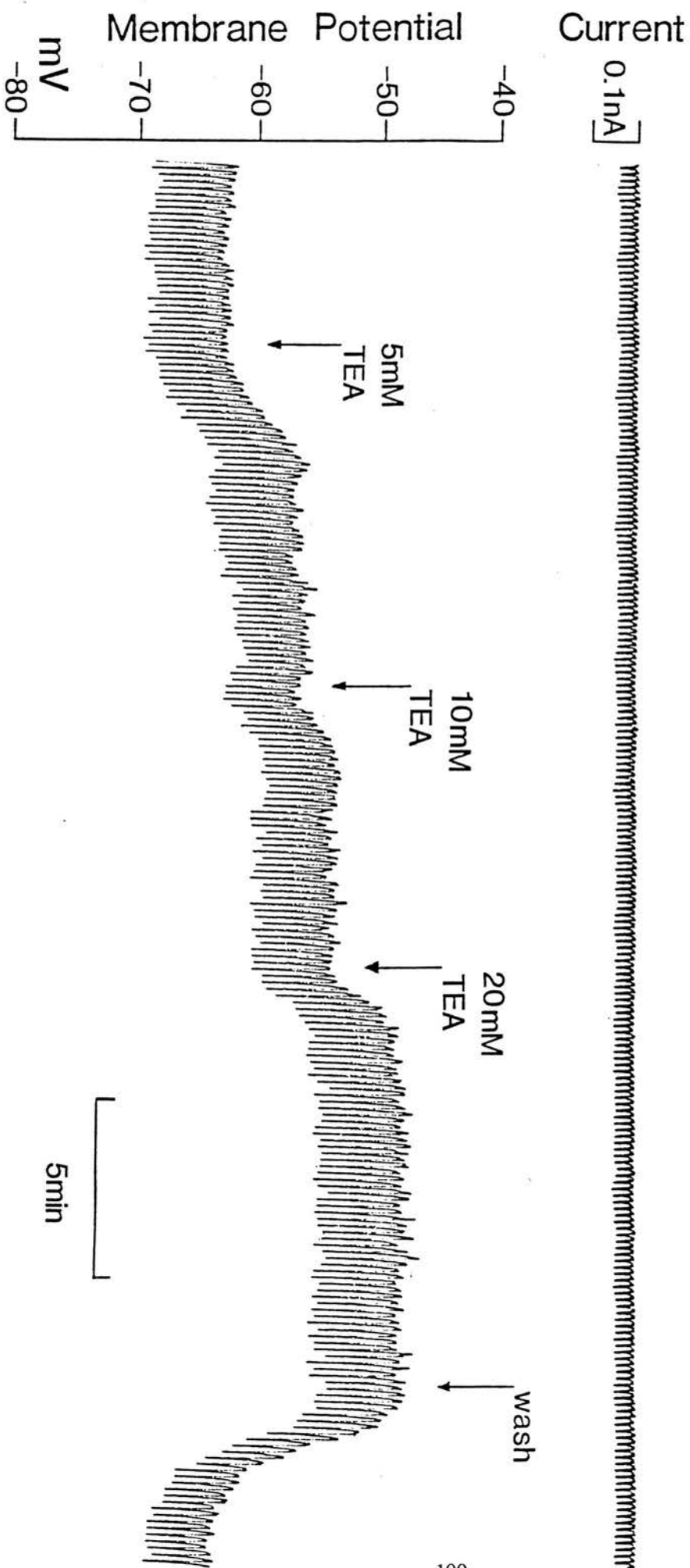


Figure 5.5. Effect of tetraethylammonium (TEA) on the hyperpolarisation induced by high pH. Raising the concentration of TEA (5, 10 and 20 mM) produced step-wise reductions in the amplitude of the pH_o -induced hyperpolarisation. The effects of TEA were reversed by washing with normal solution (pH 9.5). Membrane resistance change was monitored by observing the injected current (upper trace) and the membrane potential (lower trace).

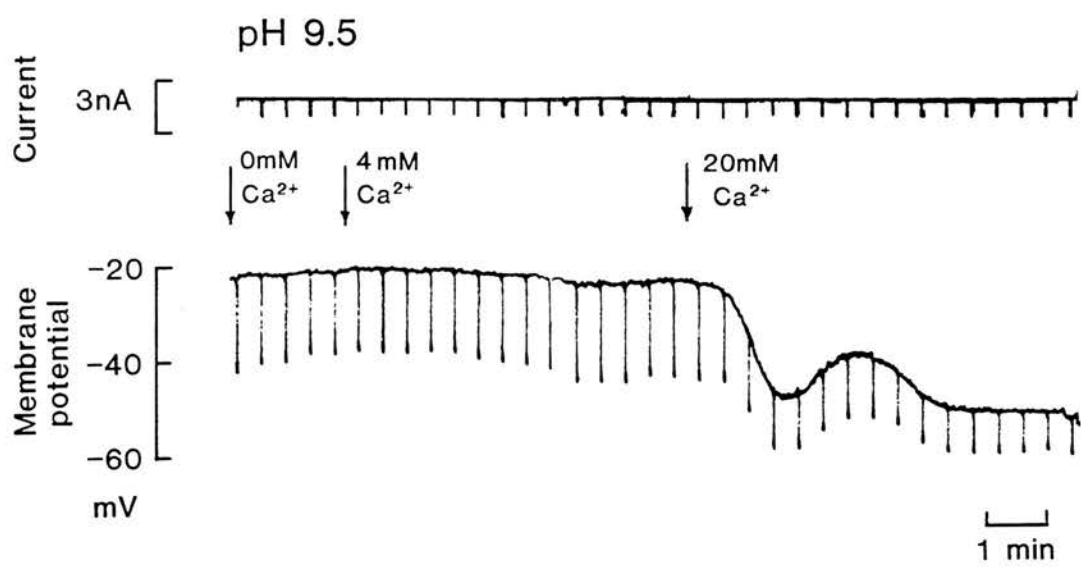


Figure. 5.6. Effect of external calcium on the initiation of the hyperpolarising response. Arrows indicate the time when the perfusion system was changed in the bath. Membrane resistance change was monitored by observing the injected current (upper trace) and the membrane potential (lower trace).

This suggests that a transmembrane Ca^{2+} flux is involved in the initiation of the response.

If maintenance of the pH_o -induced hyperpolarisation depended on Ca^{2+} influx, then reducing $[\text{Ca}^{2+}]_o$ would be expected to cause a corresponding reduction in the amplitude of the hyperpolarisation. Indeed this was found to be the case in seven experiments; the amplitude of the hyperpolarisation induced by high pH_o was dependent on $[\text{Ca}^{2+}]_o$. A representative example of one experiment is shown in Fig.5.7; the hyperpolarisation was evoked by 20 mM Ca^{2+} at pH_o 9.5 and the Ca^{2+} concentration reduced to 10, 4, 2 and 1 mM (still at pH 9.5).

In contrast, at pH_o 7.5, the membrane potential was not very sensitive to changes in $[\text{Ca}^{2+}]_o$ as is evident from Table 5.1. This is illustrated in Fig.5.8, where the sensitivity of the membrane potential to the external Ca^{2+} concentration is plotted for cells at pH 7.5 and 9.5. In each experiment, raising the value of $[\text{Ca}^{2+}]_o$ was associated with an increase in the amplitude of the hyperpolarisation induced by pH 9.5. The mean values of the membrane potential at high pH_o for a $[\text{Ca}^{2+}]_o$ of 2 and 20 mM were -53 ± 17 (n=3) and -64 ± 17 mV (n=6).

A pH_o -induced hyperpolarisation was not sustained in cells where extracellular Ba^{2+} replaced Ca^{2+} (three experiments). Responses to high pH_o were recorded, however, when Ca^{2+} was substituted by Sr^{2+} (five experiments). Although Sr^{2+} could be used as a substitute for Ca^{2+} , the Sr^{2+} concentration had to be raised to 10 mM before a pH_o -induced hyperpolarisation could be sustained, whereas Ca^{2+} was effective at 4 mM. The value of E_R for responses sustained by Sr^{2+} was -78 ± 5 mV and the increase in mean membrane conductance was 7.8 nS. Both values are similar to those for responses sustained by Ca^{2+} .

E Intracellular application of EGTA

The results so far suggest that the pH_o -induced hyperpolarisation is caused by the opening of K^+ channels probably activated by a rise in the cytosolic free Ca^{2+} concentration. A test, albeit indirect, of this hypothesis is to load eggs with the Ca^{2+} chelator EGTA (see Section 2) before treatment with high pH_o . This should prevent a rise in the cytosolic free Ca^{2+} concentration.

In five experiments, loading the eggs with EGTA prevented a pH_o -induced hyperpolarisation. Moreover, trains of action potentials in the form of anode break responses (see Section 6) could not produce hyperpolarisations in EGTA-loaded eggs (n=5). Because the EGTA electrodes had low resistance, it was necessary to investigate the effect of the insertion of low resistance electrodes on the pH-induced response.

Current

0.05nA]



Membrane Potential

mV

-50
-60
-70
-80

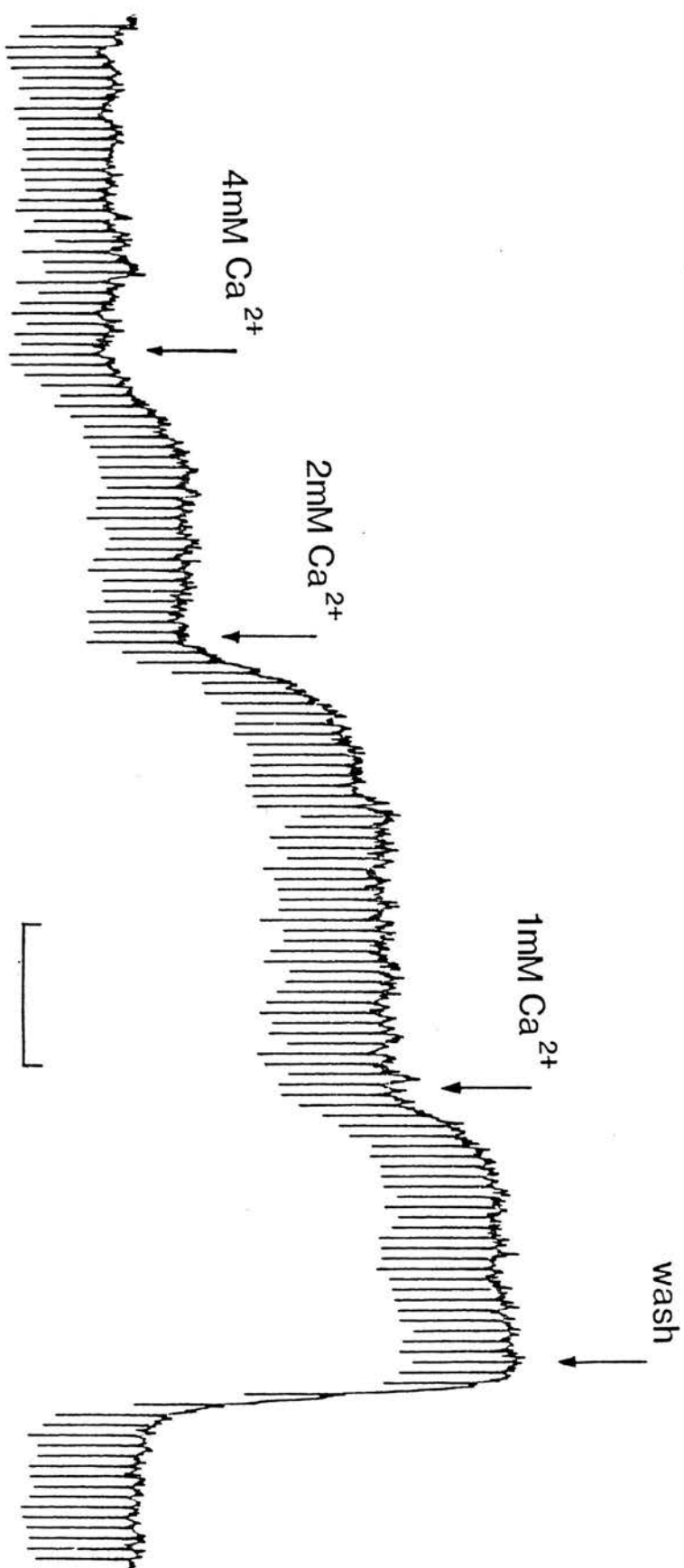


Figure. 5.7. Effect of external calcium on the maintenance of the pH_o -induced hyperpolarisation.

A reduction in the external calcium concentration at the time indicated by the arrows decreases the amplitude of the pH_o -induced hyperpolarisation. Returning the external calcium concentration to 20 mM returns the membrane potential to near its original value.

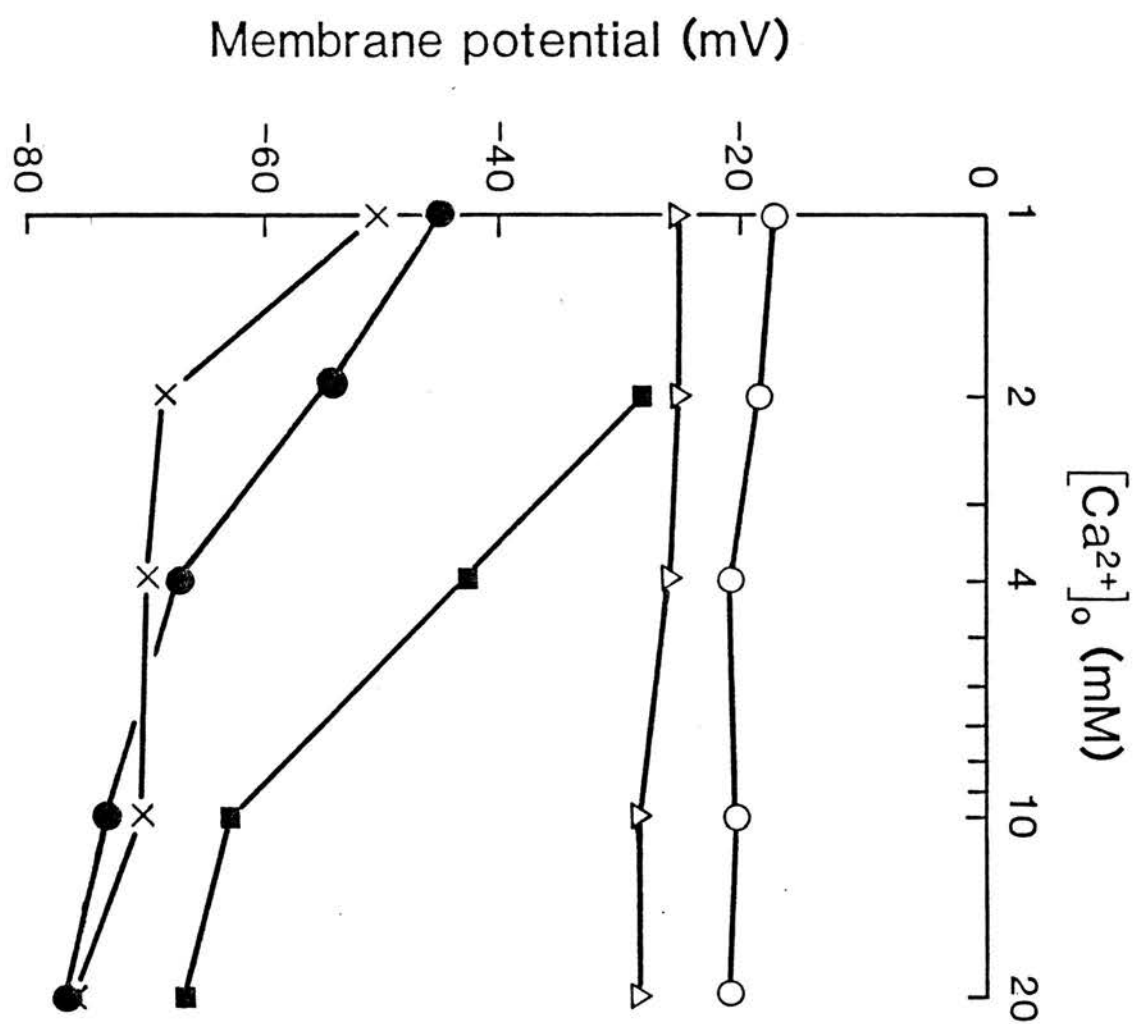


Figure 5.8. The effect of the external calcium concentration on the membrane potential at pH 7.5 and at high pH. Open triangles and circles show values obtained at pH 7.5, and closed squares, closed circles and crosses indicate values at pH 9.5. The values were obtained from five different cells.

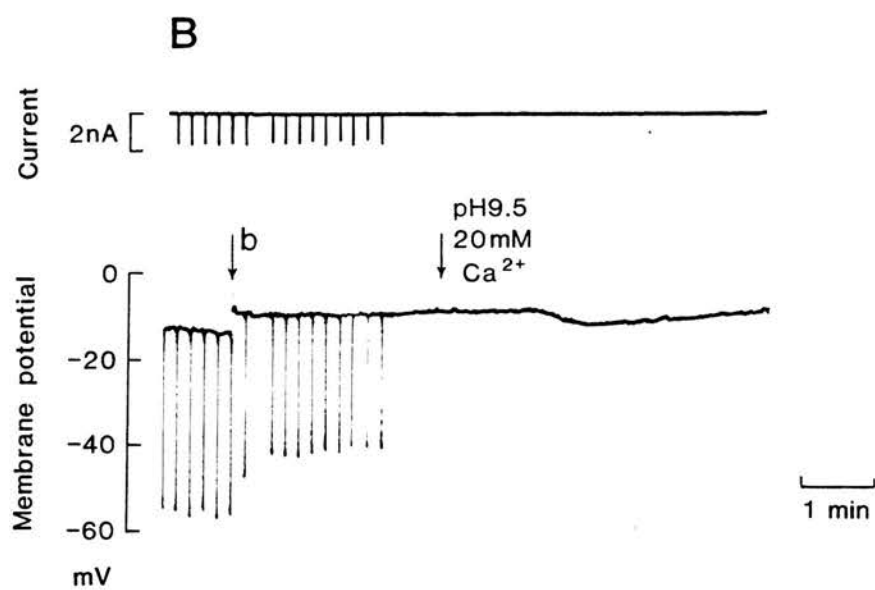
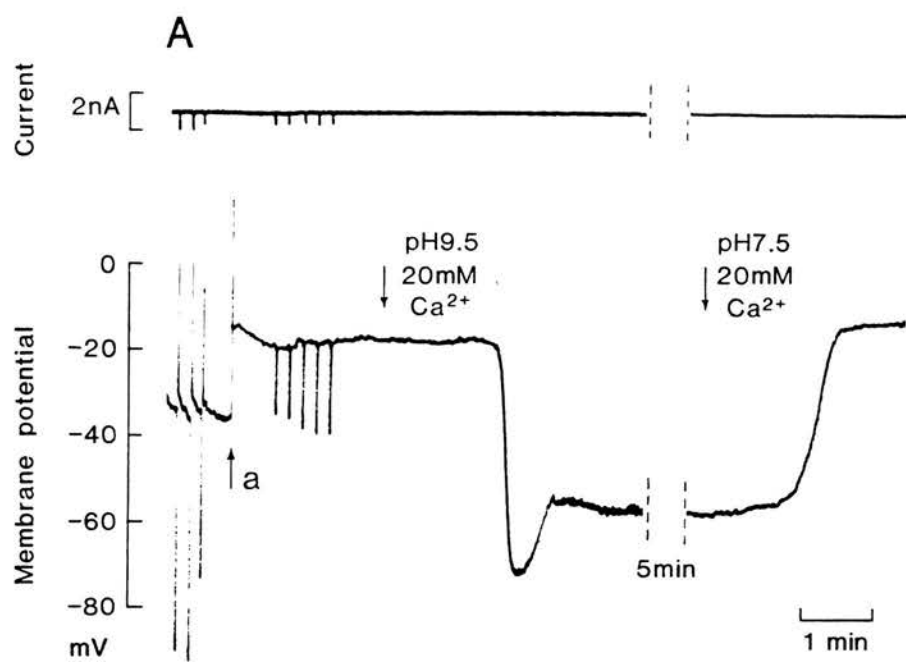


Figure 5.9. Effect of an intracellular application of the calcium chelator EGTA to an egg. Upper trace, current. Lower trace, membrane potential. **A**, a second electrode filled with potassium acetate was inserted into the egg (a) before the applications of high pH. **B**, a third electrode filled with 0.25 M EGTA was introduced into the egg (b) after the withdrawal of the second. High pH solution was then applied to the egg. Electrotonic potentials were applied at intervals to monitor the input resistance.

In a typical experiment a second low-resistance electrode filled with K⁺ acetate was inserted into an egg (a, Fig.5.9 A) to mimic the insertion of an EGTA electrode prior to application of high-pH solution. A normal response to high pH_o was obtained, and the egg washed with pH_o 7.5 solution (second arrow, Fig.5.9 A) to obtain recovery of the membrane potential. The second electrode was removed and an EGTA electrode was inserted (b, Fig.5.9 B). After the insertion of the EGTA electrode, there was sufficient leakage of EGTA into the cell to prevent a conspicuous hyperpolarisation by high pH_o (second arrow, Fig.5.9 B).

By applying an equivalent circuit model it is possible to estimate the membrane potential attained on the opening of K⁺ channels by high pH_o. For example, in Fig.5.9 A the resting potential (E_M) is -20 mV and the conductance (g) is 4.5 nS. The opening of K⁺ channels by high pH_o causes an expected increase of conductance (g_K) of 6.6 nS with an E_R = -85 mV (Table 5.1). The expected potential at high pH_o is given by:

$$\frac{E_M g + E_R g_K}{g + g_K} = \frac{(-20)(4.5) + (-85)(6.6)}{4.5 + 6.6} = -59 \text{ mV}$$

In Fig.5.9 B, E_M = -14 mV and g = 5.3 nS and so the expected potential at high pH_o is given by:

$$\frac{(-14)(5.3) + (-85)(6.6)}{5.3 + 6.6} = -52 \text{ mV}$$

Thus any increase in the leak impalement artifact produced by the insertion of the EGTA pipette is insufficient to account for the failure of high-pH solution to evoke a hyperpolarisation.

F Is passive Ca²⁺ influx through voltage-gated Ca²⁺ channels responsible for the pH_o-induced hyperpolarisation ?

Voltage clamp studies (Okamoto, Takahashi & Yamashita, 1977) and intracellular recordings (Miyazaki and Igusa, 1982; Georgiou, Bountra, Bland & House, 1983; Yoshida, 1983) have revealed the presence of voltage-gated Ca²⁺ channels in both mouse and hamster eggs.

These channels are normally inactivated at the resting potentials recorded by intracellular micro-electrode (around -30 mV). It was possible, however, that the high pH_o could increase their open state probability at these potentials and so cause an enhancement of the passive Ca²⁺ influx.

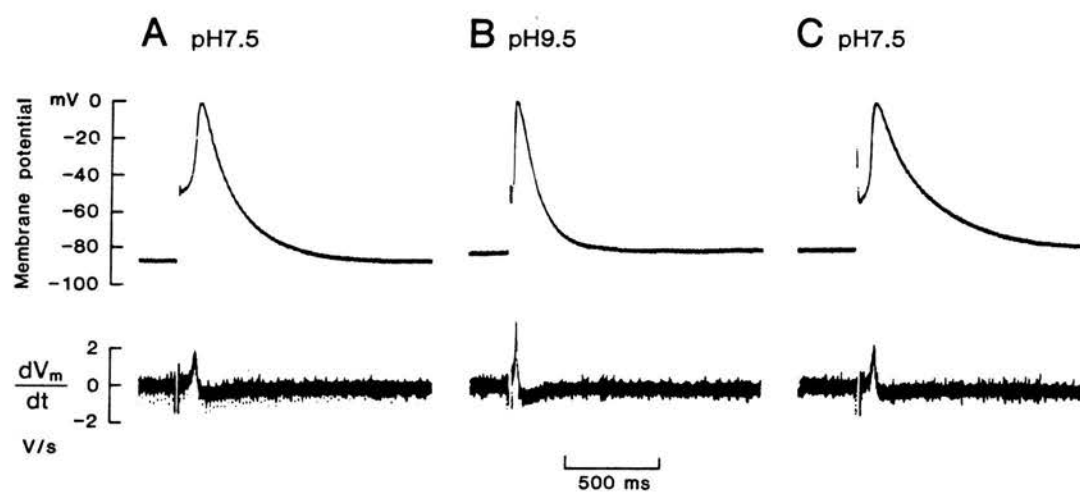


Fig. 5.10. Effects of high external pH on calcium action potentials in a hamster egg current-clamped at a membrane potential (V_M) of about -90 mV. Oscilloscope pictures are presented of action potentials (upper) and their differentiated records (lower) in standard solution (A), in high pH_o solution (B) and after washing with standard solution (C).

An indirect estimate of the Ca^{2+} influx through the voltage-gated channels is obtained by measuring the maximum rate of rise (MRR) of the Ca^{2+} action potential. To obtain such estimates, cells were current-clamped at membrane potentials in the range -90 to -140 mV and the action potentials evoked by depolarising pulses in 4 mM Ca^{2+} solution were electronically differentiated. An example of action potentials evoked in varying pH_o and their differentiated signals is illustrated in Fig.5.10.

The mean MRR value at $\text{pH}_o = 7.5$ was 1.7 ± 0.2 V/s ($n=12$). The MRR of Ca^{2+} action potentials was reversibly increased to 2.8 ± 0.6 V/s at $\text{pH}_o = 9.5$ in the same cells. In addition, the threshold of the action potential shifted to more negative values by about 4mV. This negative shift in threshold at high pH_o further excludes the participation of the voltage-gated Ca^{2+} channels in the response to pH_o .

The rate of repolarisation of the Ca^{2+} action potentials was also reversibly increased by the high-pH treatment in the same cells. The mean values of maximum rate of fall were -0.24 ± 0.10 V/s at pH 7.5 and -0.42 ± 0.15 V/s respectively ($n=12$).

A further test of the possible role of the voltage-gated Ca^{2+} channels in the initiation and maintenance of the response to high pH_o was carried out by adding the Ca^{2+} -channel blocker, La^{3+} to the bathing solution in eight experiments. The action potentials were tested for by shifting the membrane potential to negative values by injecting d.c. current; depolarising current pulses failed to evoke action potentials in the presence of La^{3+} . The presence of La^{3+} did not prevent the response to high pH_o , suggesting that the voltage-gated Ca^{2+} channels are not a major route for the necessary Ca^{2+} influx. Because La^{3+} is also an inhibitor of the Ca^{2+} pump it was used for that purpose in experiments in Section 4.

G Is the pH_o -induced response mediated by a rise in intracellular pH_i ?

It was possible that raising the extracellular pH would produce a rise in pH_i , and that this rise in pH_i was responsible for evoking the hyperpolarisation. A rise in pH_i has been shown to cause a rise in $[\text{Ca}^{2+}]_i$ in Aplysia neurones (Zucker, 1981) on application of NH_4Cl . This chemical is known to cause internal alkalinisation, as is trimethylamine (Thomas, 1984). Both agents were applied externally to the hamster egg in a normal solution of pH 7.5. Even after 40 min of bathing the egg with 20 mM NH_4Cl (four eggs) or 20 mM Trimethylamine (three eggs), no hyperpolarisation occurred suggesting that internal alkalinisation may not be the mechanism for the pH_o effect.

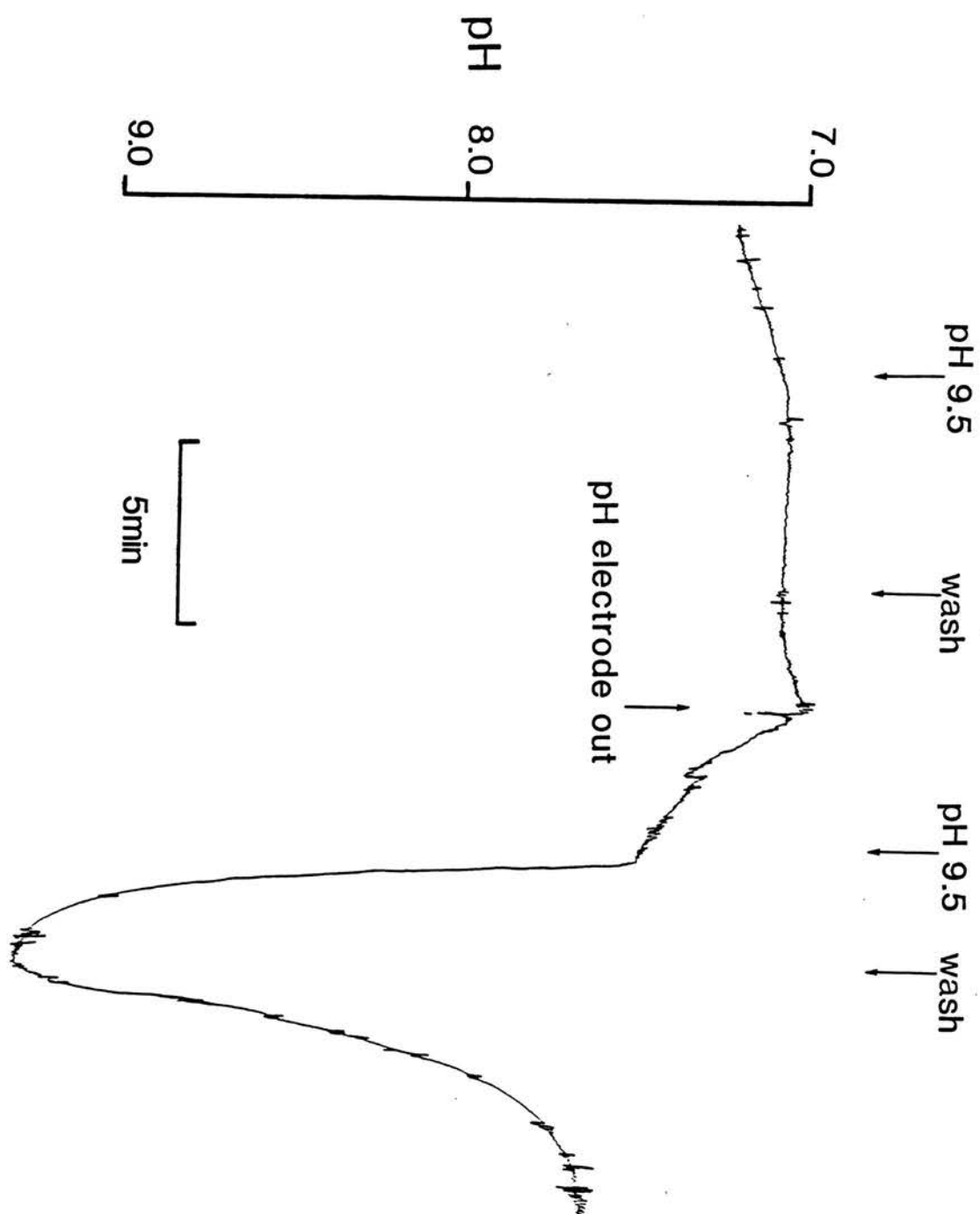


Fig. 5.11. Measurement of pH_o using a pH-sensitive electrode. With the pH electrode inside the egg, raising pH_o to 9.5 had little effect on pH_i . Removal of the pH-electrode from the egg showed that it was sensitive to a rise in the pH of the bathing solution.

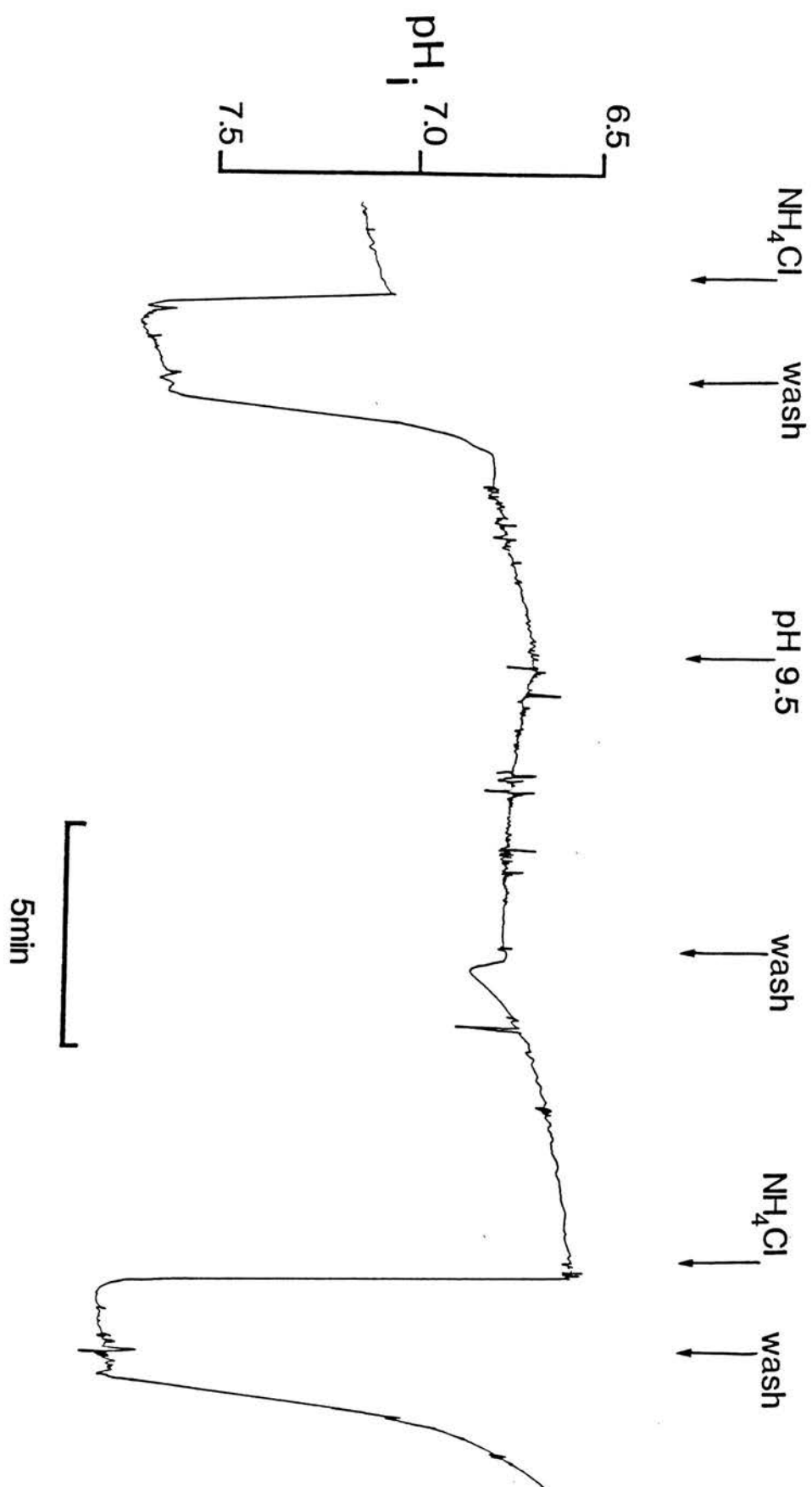


Fig. 5.12. Comparison of the effects of raising pH_o and application of NH_4Cl on pH_i , using a pH-sensitive electrode. Application of 15 mM NH_4Cl in a pH 7.5 solution produced a change in pH_i (0.67 units); as soon as this was observed, the egg was washed with normal solution. High pH_o solution (9.5) was applied and little change in pH_i noted. After washing with normal solution at pH 7.5, a rise in pH_i was again seen on application of NH_4Cl at pH 7.5.

Attempts to measure intracellular pH with a pH-sensitive electrode proved difficult (see Section 2), as the electrodes available were of low resistance and often damaged the cell on penetration. Results from three cells were obtained, giving an indication that raising pH_o had very little effect on pH_i , while external application of NH_4Cl produced a dramatic rise in pH_i . The only effect of raising pH_o on pH_i observed was a slowing of the tendency of pH_i to acidify (Fig.5.11). The lack of a change in pH_i after raising pH_o was not due to a fault in the pH electrode, as it responded to pH 9.5 after being taken out of the cell.

Application of 15-20 mM NH_4Cl on the other hand, did produce an internal alkalinisation as would be expected for most cells (Thomas, 1984). A comparison of changes in pH_i induced by NH_4Cl and pH_o 9.5 is shown in Fig.5.12. The NH_4Cl evoked a change in pH_i of 0.67 (Fig.5.12 A) and 1.1 pH units (Fig.5.12 C), while pH_o 9.5 had little effect (Fig.5.12 B). This result also illustrates that the pH-sensitive electrode could respond to changes in pH_i , and that the result illustrated in Fig.5.11 was not just due to a failure of this electrode to function in the cell.

Discussion.

A K^+ conductance underlies the high pH_o -induced hyperpolarisation.

The results demonstrate that raising the pH of the solution bathing hamster eggs can produce a reversible membrane hyperpolarisation accompanied by a rise in membrane conductance. Reversal potential measurements suggest that high pH_o activates a K^+ or a Cl^- conductance. Although the presence of Ca^{2+} -activated K^+ channels has been demonstrated in mammalian eggs (Miyazaki & Igusa, 1983; Georgiou et al, 1983), Ca^{2+} -activated Cl^- channels have been reported in *Xenopus* oocytes (Barish, 1983; Miledi & Parker, 1984) so it was necessary to test for the latter type in hamster eggs. Methane sulphonate is a useful replacement anion for Cl^- as it is impermeant and does not alter intracellular pH (Sharp & Thomas, 1981).

The amplitude of the hyperpolarisation is unaffected by the removal of extracellular Cl^- , while it varies with $[K^+]_o$ (Fig.5.4), strongly suggesting that the pH-evoked response is caused by the opening of K^+ channels. This interpretation is supported by the finding that TEA partially blocks the pH_o -induced rise in membrane conductance; TEA is known to block different types of Ca^{2+} -activated K^+ channels to varying degrees (see Hermann and Hartung, 1986 for review).

The discrepancy between the observed relation between potential and $\log [K^+]_o$ in Fig.5.4 and that predicted by the Nernst equation implies the presence of an additional conductance pathway. The deviation of the relation from linearity at concentrations of K^+ below 5 mM is probably due to

the increase in the significance of the contribution of Na^+ , as reported for squid giant axons (Hodgkin and Keynes, 1955).

Dependence of the response on Ca^{2+} .

Calcium ions are required for the initiation and maintenance of the pH_o -induced hyperpolarisation. The amplitude of the response decreased when $[\text{Ca}^{2+}]_\text{o}$ was lowered and was abolished when external Ca^{2+} was replaced by magnesium or barium. Moreover, strontium was an effective substitute for Ca^{2+} . Igusa & Miyazaki (1983) reported the same order of ionic dependency for mediating the hyperpolarisations occurring after fertilisation (in both the fertilisation events and the pH_o -induced response, Sr^{2+} is less effective than Ca^{2+} at equimolar concentrations). The results are also compatible with those for intracellular cation injections (Section 3).

The possibility that the effects of decreasing $[\text{Ca}^{2+}]_\text{o}$ on the membrane potential and input resistance of the egg was due to a reduction in seal resistance, rather than an altered Ca^{2+} influx, seems unlikely; decreasing $[\text{Ca}^{2+}]_\text{o}$ at pH 7.5 has little effect on these parameters (Fig.5.8). The dependence of the pH_o -induced response on $[\text{Ca}^{2+}]_\text{o}$ suggests that an influx of Ca^{2+} is necessary to initiate and sustain it. A similar conclusion was reached by Igusa & Miyazaki (1983) regarding the hyperpolarisations after fertilisation.

A rise in $[\text{Ca}^{2+}]_\text{i}$ at fertilisation has been recorded and shown to underly these recurring hyperpolarisations (Igusa & Miyazaki, 1986). If the same K^+ channels are opened by high pH_o , then preventing this rise in Ca^{2+} should abolish the hyperpolarisation, as found when EGTA was injected into fertilised eggs (Miyazaki & Igusa, 1982). In the experiments above (E, Section 5), the insertion of the EGTA-filled pipette might introduce an additional leak conductance which, if substantial, could itself reduce the amplitude of the pH_o -induced hyperpolarisation.

The insertion of a similar low-resistance pipette (filled with potassium acetate) in place of the EGTA pipette, however, failed to attenuate the response (Fig.5.9A). Thus the abolition of the response by the intracellular application of EGTA (Fig.5.9B) appears to be genuine and implicates intracellular Ca^{2+} as a mediator in the response to high pH_o .

Direct effects of pH_o on channels.

A possible mechanism underlying the suggested pH_o -induced rise in $[\text{Ca}^{2+}]_\text{i}$ could be a direct effect of high pH_o enhancing Ca^{2+} influx through voltage-gated Ca^{2+} channels existing in hamster eggs (Miyazaki & Igusa, 1982). Altering pH_o affects current and gating of channels in skeletal muscle (Hille, 1986; Campbell & Hahn, 1984). Although high pH_o did directly affect voltage-gated Ca^{2+} channels in the egg (Fig.5.10), preventing influx through these channels by lanthanum

did not block the response, indicating that they do not underly the hyperpolarising response. Additionally, raising the pH_o shifted the threshold for activation of the channels further away from the recorded resting potential. This would suggest that the influx of Ca^{2+} required to sustain this response is through non-voltage gated Ca^{2+} channels.

It is possible that raising pH_o has a direct effect on Ca^{2+} -mediated K^+ channels. However, Light, Van Eenenaam, Sorenson & Levitt (1987) reported no change in the behaviour of Ca^{2+} -mediated K^+ channels between pH 6.1 and 8.0 in hamster insulin tumour cells. Single channel analysis is necessary to determine if there is any effect of pH on the behaviour of these channels in the hamster egg.

The site of action of high pH_o .

To examine the possibility that the pH_o -induced response was caused by a rise in intracellular pH, the effect of extracellular application of trimethylamine and NH_4Cl was investigated. These chemicals are known to produce a rise in pH_i in other preparations (Zucker, 1981; Thomas, 1984). Additionally, NH_4Cl is known to parthenogenetically activate sea urchin eggs (Shen, 1982; Whitaker and Steinhardt, 1982) and trigger protein synthesis independent to the rise in pH_i (Dube and Epel, 1986). Neither NH_4Cl ($n=4$ eggs) or trimethylamine ($n=3$ eggs) produced a hyperpolarisation in the hamster egg.

It was found that NH_4Cl did produce a rise in pH_i (Fig.5.12, A & C), but it was possible that this rise was not as large as the one produced by pH_o 9.5. However, when the effect of raising pH_o to 9.5 was measured, it was found to have little effect on pH_i (Figs.5.11 A & 5.12 B).

Consistent with these results is the hypothesis that the action of the high pH_o was mediated at an external site on the membrane, as internal alkalisation by NH_4Cl did not evoke a hyperpolarisation or rise in membrane conductance. This is also suggested by the rapid onset (as fast as 6 s) and offset of the response when the high pH was directly applied to the egg (Fig.5.3, B & C).

In conclusion, the results of this section show that raising the pH of the solution bathing the hamster egg activates an increased K^+ conductance. This conductance seems to be dependent on an influx of Ca^{2+} across the egg membrane through non-voltage gated Ca^{2+} channels, leading to an increase in $[Ca^{2+}]_i$.

SECTION 6 Evidence for calcium stores in the hamster egg.

The Ca^{2+} -activated K^+ channels of hamster eggs are activated by a rise in $[\text{Ca}^{2+}]_i$ during fertilisation; transient $[\text{Ca}^{2+}]_i$ rises occur from basal ($0.4 \mu\text{M}$) to peak ($1 \mu\text{M}$), activating these channels to produce a series of hyperpolarisations (Igusa & Miyazaki, 1983 & 1986). The mechanism by which the sperm produces these rises in $[\text{Ca}^{2+}]_i$ is unknown, but Igusa and Miyazaki (1983) have proposed the involvement of Ca^{2+} -induced Ca^{2+} release from intracellular stores. Recently, Miyazaki (1987) showed that injection of the second messenger inositol 1,4,5-trisphosphate (IP_3) and the hydrolysis-resistant analogue of GTP (GTPyS) produced a rise in $[\text{Ca}^{2+}]_i$ in the hamster egg.

The results presented in this section provide evidence to support the existence of a mechanism that produces an amplified rise in the $[\text{Ca}^{2+}]_i$ in the unfertilised egg either by Ca^{2+} -induced Ca^{2+} release or via second messengers.

The lines of evidence are, 1) that a single action potential can produce a large transient hyperpolarisation after a delay (ca. 9 s) and 2) that a sigmoid curve is obtained when the relation between the size of a Ca^{2+} injection pulse and the size of the Ca^{2+} -evoked hyperpolarisation is plotted.

Results.

A Influx of Ca^{2+} through voltage-gated Ca^{2+} channels.

Repetitive Ca^{2+} action potentials can produce a rise in $[\text{Ca}^{2+}]_i$ activating a K^+ conductance. To enhance influx through the Ca^{2+} channels, recordings were made in solutions containing 20 mM CaCl_2 . Repetitive stimulation (frequency, 1.0-1.2 Hz; pulse duration, 500 ms) of an egg at an external pH (pH_o) of 7.5 produced a transient hyperpolarisation out-lasting the stimulation period. An example of this is illustrated in Fig.6.1, where the hyperpolarisation is accompanied by an increase in input conductance. This conductance increase is probably mediated by K^+ ; the estimated reversal potential for the transient hyperpolarisation was $-89.0 \pm 8.2 \text{ mV}$ ($n=3$).

Generally a second similar response could not be evoked even after a resting period of 20 min; instead hyperpolarisations graded in size with the number of action potentials were obtained (Fig.6.2).

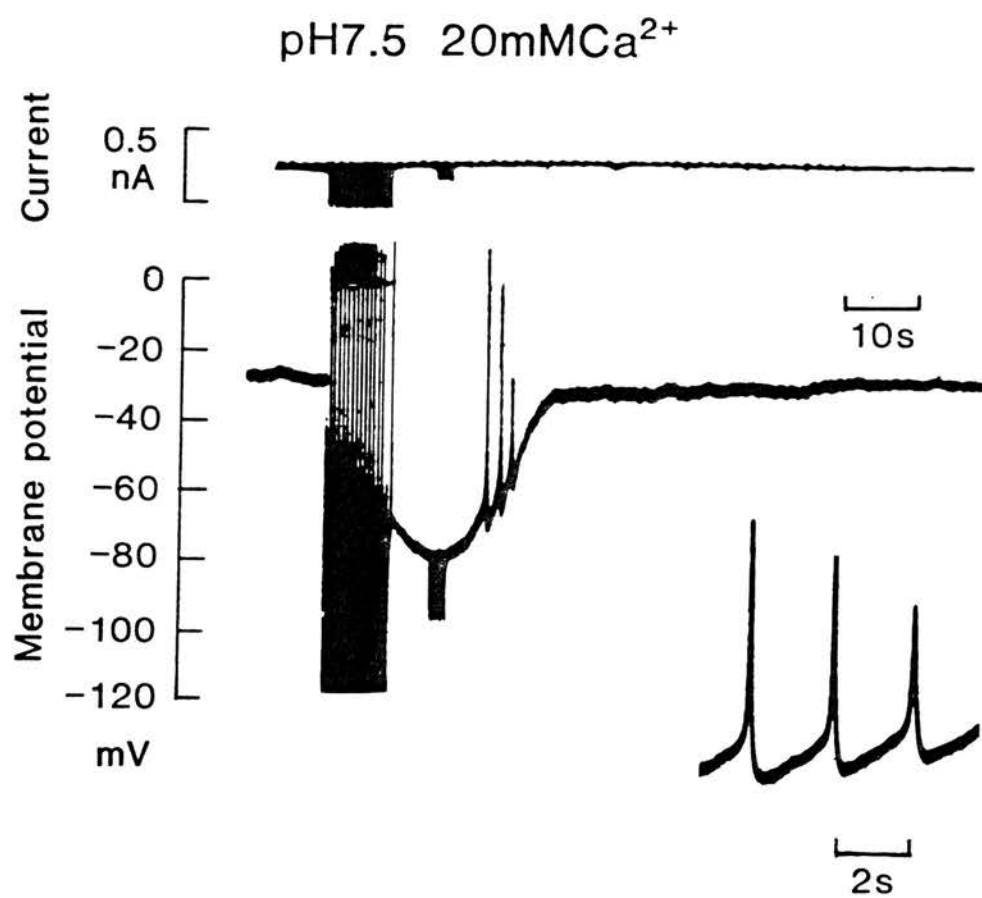


Figure 6.1. A transient hyperpolarisation of an egg at an external pH (pH_o) of 7.5, induced by repetitive hyperpolarising pulses leading to anode-break responses (1.0-1.2 Hz). Some action potentials were observed on the recovery phase of the hyperpolarisation, probably due to activation of voltage-gated calcium channels. The hyperpolarisation out-lasts the stimulation period. Upper trace, current pulses; lower trace membrane potential

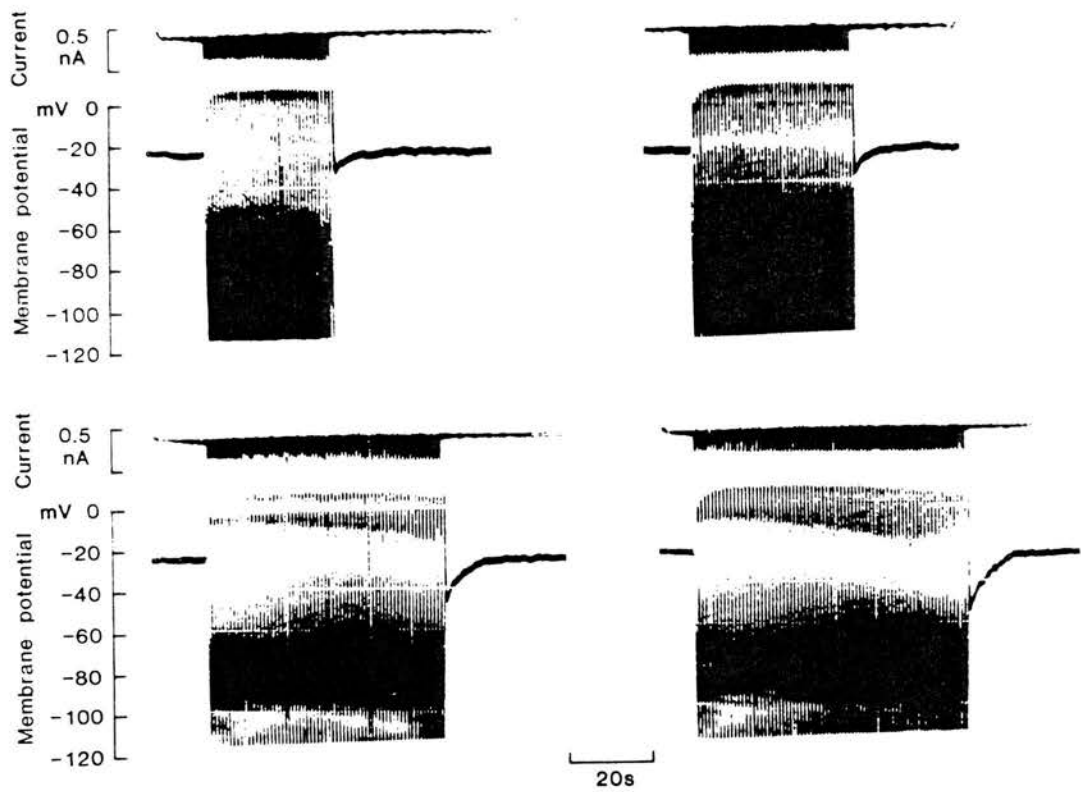


Figure 6.2. Repetitive hyperpolarising pulses leading to anode-break responses (1.0-1.2 Hz) fail to evoke a second response similar to the one illustrated in Figure 6.1 once this first response was evoked. This was the case even after a resting period of 20 min, despite increasing the number of stimuli.

B A delayed all-or-none hyperpolarisation induced by a single Ca^{2+} action potential.

A transient large hyperpolarisation with a delay of 9.0 ± 4.0 s ($n=8$) could be triggered by a single Ca^{2+} action potential in some cells. In total, 8 out of 33 cells displayed this response; the remaining cells all responded to three or more action potentials in succession with a hyperpolarisation. Again, the Ca^{2+} concentration of the solution bathing the egg was raised to 20 mM to enhance Ca^{2+} influx in these experiments.

In some cases, the external pH was raised to 8.0 to inhibit Ca^{2+} efflux (Georgiou, House, McNiven & Yoshida, 1988), enhancing the expected rise in $[\text{Ca}^{2+}]_i$. Figure 6.3 shows a delayed hyperpolarisation evoked by a single anode-break response in a cell at the resting potential. In this example a latency of 7.0 s was observed between the anode break excitation and the induction of the hyperpolarisation. A second anode-break responses soon after the hyperpolarisation failed to produce another hyperpolarisation. In two cells however, a second transient response could be evoked by a second action potential. However, these responses had a much smaller amplitude and delay than the first action potential induced hyperpolarisation. In one of these cells, a latency of 17.6 s was observed between the first anode-break excitation and the induction of the hyperpolarisation (Fig. 6.4A). For the second response, the latency between the action potential and the hyperpolarisation was 4 s (Fig.6.4B).

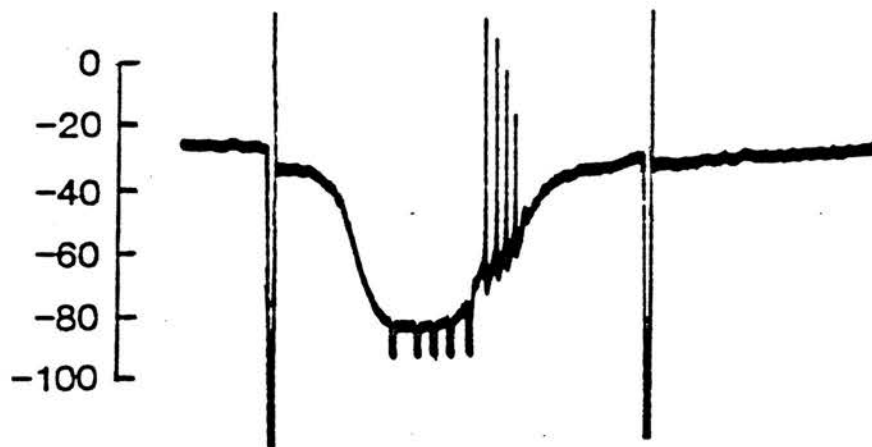
After this second response (and after the single response in the other six cells), hyperpolarisations were graded in amplitude and duration according to the number of evoked anode-break excitations (Fig. 6.5 A, B and C). These hyperpolarisations were obtained 71 (A), 73 (B) and 76 (C) minutes after the first transient hyperpolarisation (A).

The delayed response could be reversed to a depolarisation in cells with potentials shifted by d.c. current injection to more hyperpolarised values than E_K (Fig.6.6A). Again, a second action potential failed to evoke a similar depolarising response; graded depolarising responses to trains of action potentials were obtained in current-clamped cells even 30 min after the delayed response was induced (Fig.6.6 B & C).

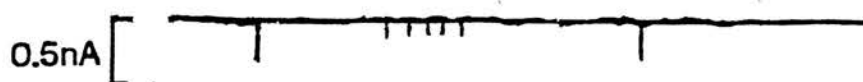
Thus some change occurred after the activation of the initial delayed response preventing the full expression of a second response to a single action potential.

In six experiments on cells with resting potentials and input resistances of -25.8 ± 2.8 mV and 472.5 ± 124.6 M Ω respectively, values at the peak of the large delayed response of -76.8 ± 9.1 mV and 103.6 ± 33.0 M Ω were recorded. The reversal potential for these responses was -86.5 ± 4.3 mV ($n=6$), compatible with the equilibrium potential for K^+ .

Membrane potential (mV)



Current



30sec

Figure 6.3. A single anode-break response produces a large transient hyperpolarisation with a delay. The hyperpolarisation was accompanied by an increase in conductance (monitored by the electrotonic potentials at the peak of the response). Action potentials occur on the recovery phase of the response. A second action potential soon after the hyperpolarisation was unable to evoke a second hyperpolarisation.

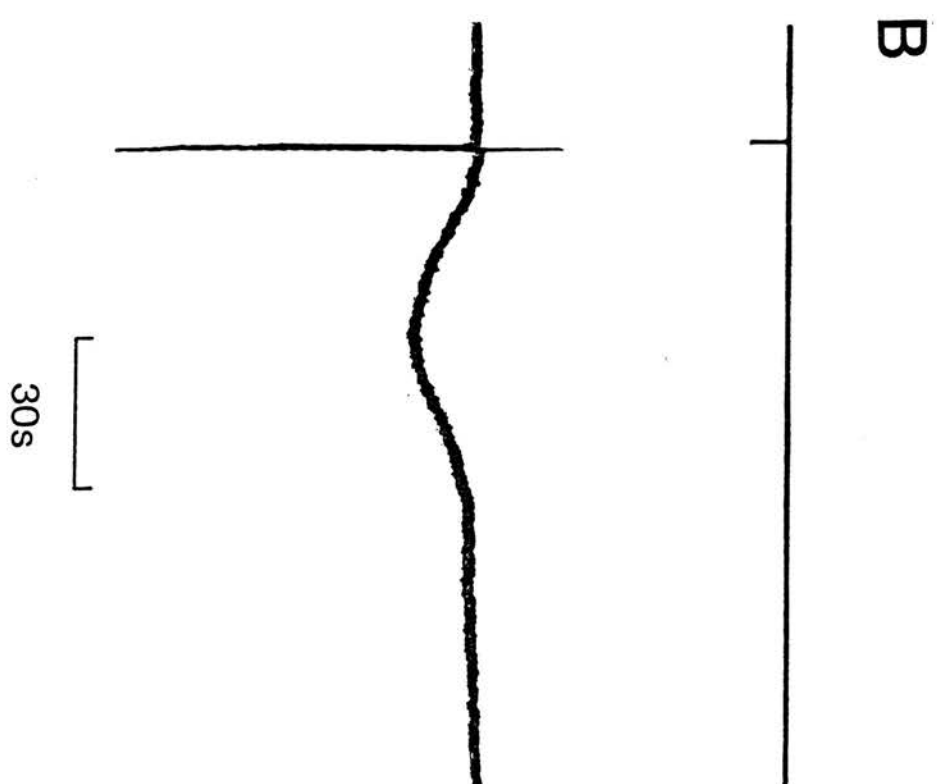
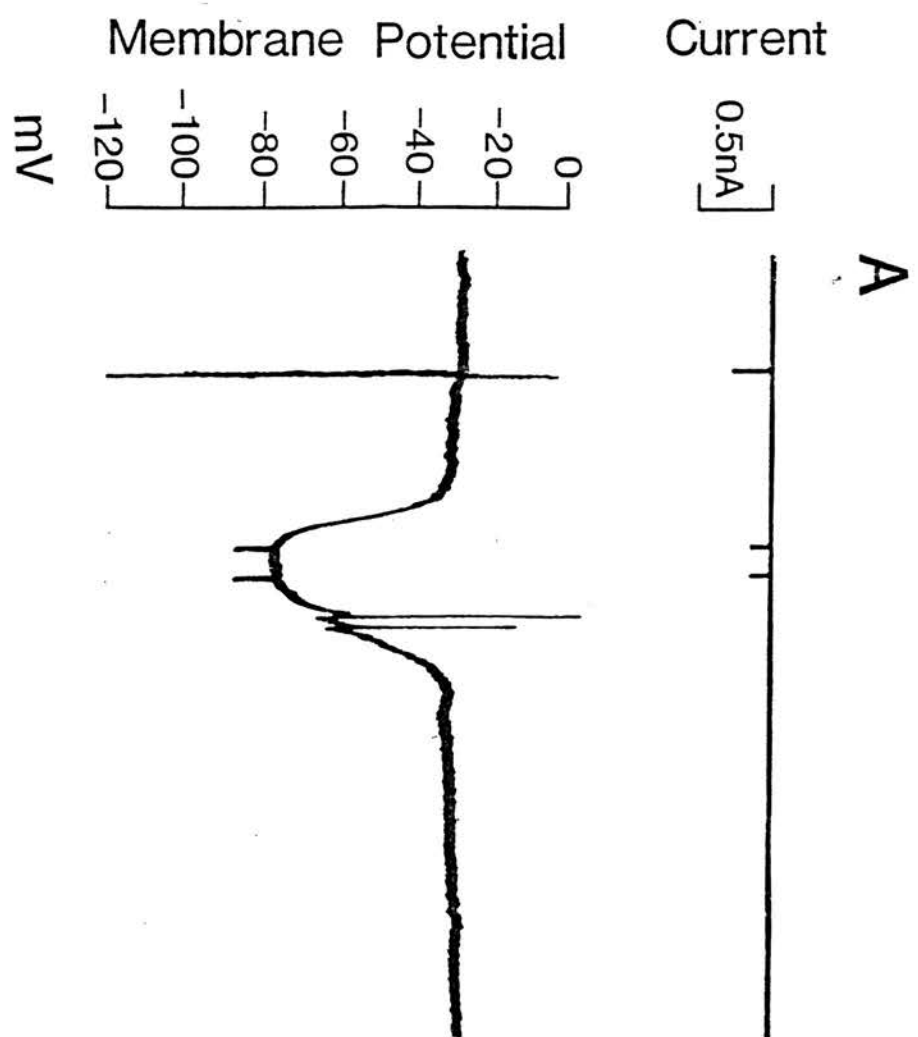


Figure 6.4. A response to a second action potential after a transient all-or-none response was rarely seen. **A**, a single action potential evokes a large hyperpolarisation with a long delay. Electrotonic potentials at the peak of the hyperpolarisation monitor the input resistance. **B**, the response to a second action potential was followed by a second smaller response with less of a delay.

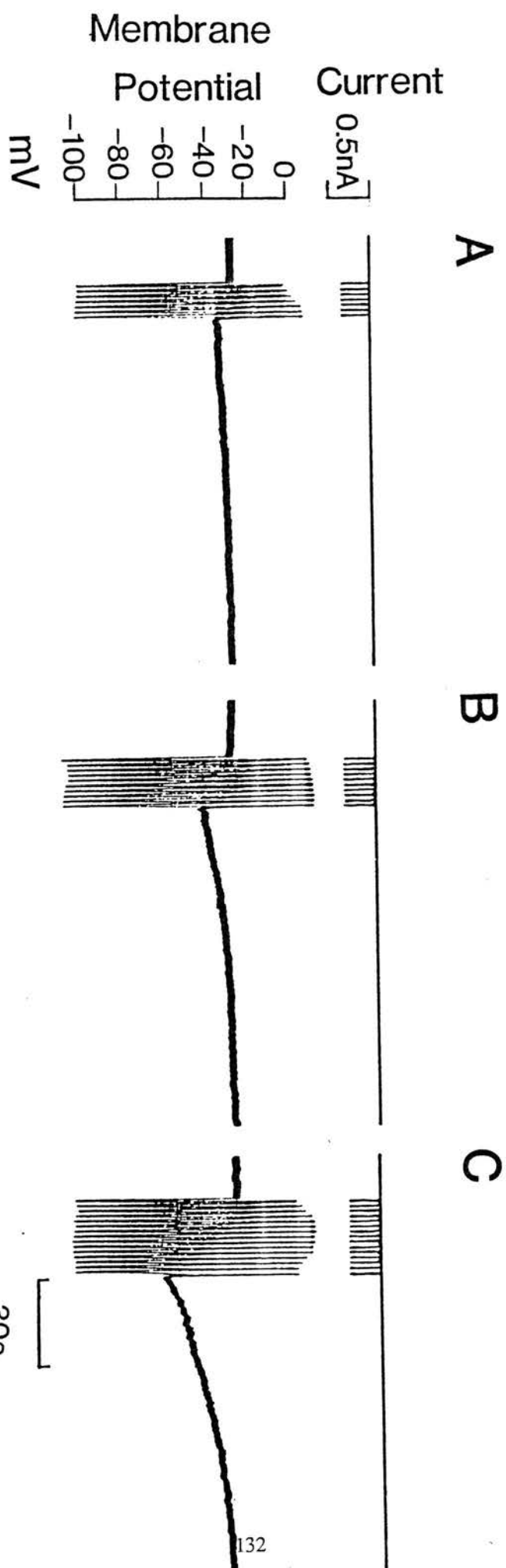


Figure 6.5. After the first two responses to single action potentials (Fig. 6.5), hyperpolarisations graded in size with the number of stimuli could be evoked up to 76 min after the first hyperpolarisation (A, B and C).

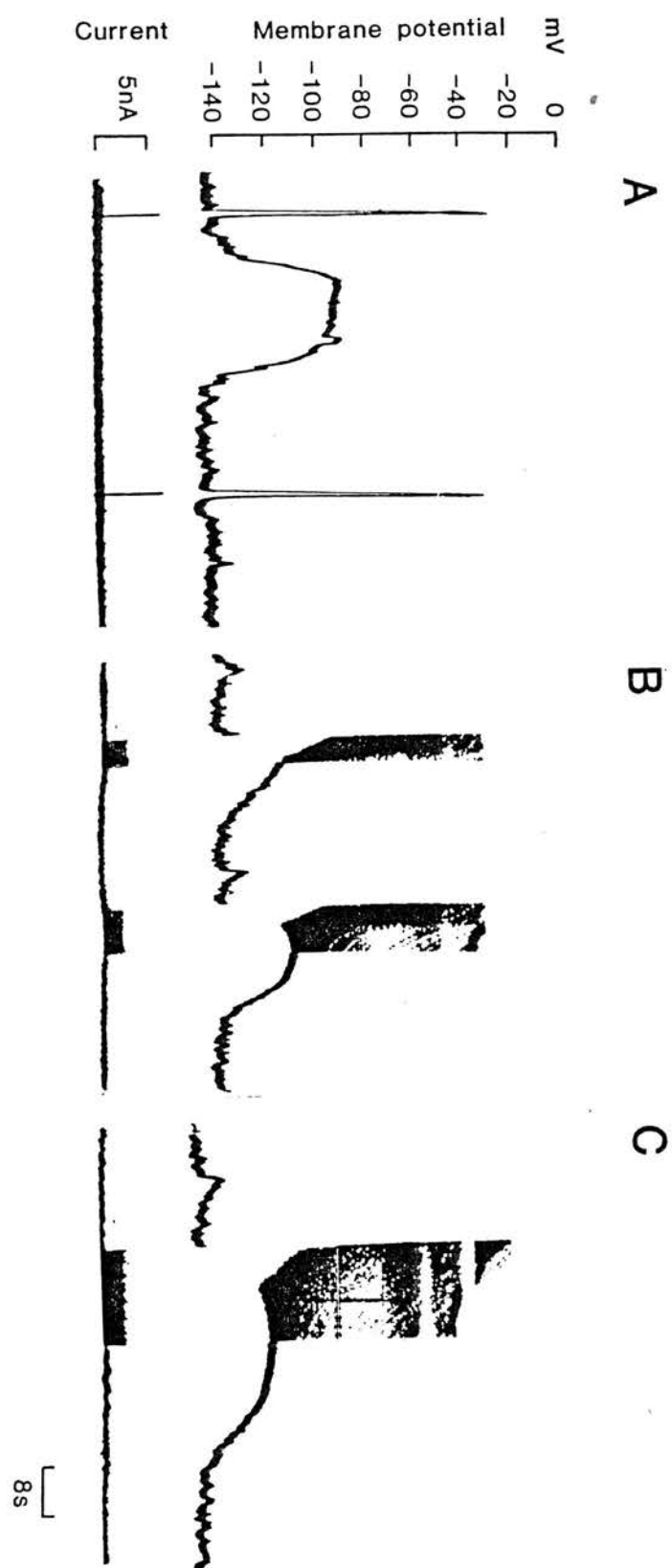


Figure 6.6. A transient potassium conductance was evoked in cells held at different potentials to the resting potential. **A**, A single action potential produces a depolarisation in a cell current-clamped to around -140 mV. Again, a second action potential soon after the depolarising response did not evoke a second response. **B**, a series of action potentials produce graded depolarisations in the cell after this. The lower trace shows the depolarising current pulses passed through the recording micro-electrode. The upper trace is the membrane potential hyperpolarised by a steady d.c. current passed through this electrode.

Evidently the Ca^{2+} influx associated with a single action potential is sufficient to lead to a large rise in the Ca^{2+} -activated K^+ conductance. Igusa & Miyazaki (1983) reported a response of the same characteristics after an injection of Ca^{2+} in the hamster egg, i.e. a 'regenerative' hyperpolarisation. There was a refractory period associated with the hyperpolarisation they report; a regenerative hyperpolarisation cannot be obtained during this period. They propose that this is due to the time required for intracellular Ca^{2+} stores to restock.

C A transient rise in K^+ conductance with a delay due to raising the pH_o to 8.0.

In 8 out of 12 cells, raising the pH_o from 7.5 to 8.0 was sufficient to evoke a transient hyperpolarisation (or depolarisation in current-clamped cells). In these cells, an action potential was not required to elicit a response. This suggests that the passive influx of Ca^{2+} in these cells was sufficient to trigger a rise in $[\text{Ca}^{2+}]_i$, due to the inhibition of Ca^{2+} efflux at pH_o 8.0 (Section 3). The four cells that did not respond to raising the pH_o to 8.0 did respond to an action potential, as discussed above in (B), producing a hyperpolarisation after a delay.

The effect of raising the pH_o on the ability of Ca^{2+} action potentials to produce a hyperpolarisation is clearly illustrated in Fig.6.7. Here, the frequency of stimulation is low enough at pH 7.5 for the Ca^{2+} efflux system to prevent a rise in $[\text{Ca}^{2+}]_i$ sufficient to hyperpolarise the cell (Fig.6.7 A & C). Raising the pH_{ov} to 8.0 results in a hyperpolarisation (Fig.6.7 B) probably due to a combined action of the pH on Ca^{2+} efflux and influx (see Section 5). This cell had previously responded to an action potential with a delayed transient hyperpolarisation.

D Suppression of the action potential-evoked hyperpolarisation by lanthanum and EGTA.

Experiments were performed to examine the possibility that the change of membrane potential per se during stimulation, without any action potential, was sufficient to evoke a delayed response or graded responses. Single hyperpolarising pulses where the anode-break response was blocked by La^{3+} (0.5 mM) did not evoke delayed transient responses: at this concentration, La^{3+} blocked action potentials but did not induce a hyperpolarisation itself (see below). Moreover, no cells loaded with the Ca^{2+} chelator EGTA could produce transient responses to single action potentials or trains of anode-break excitations ($n=8$)(Fig.6.8).

Thus the delayed response can be triggered by the rise in $[\text{Ca}^{2+}]_i$ resulting from the Ca^{2+} influx associated with a single action potential. Further evidence that the response is not due to the transient membrane hyperpolarisation occurring with the anode break response is that the response

could be evoked by an action potential which was generated by a short duration stimulation in current-clamped cells (Fig.6.6 A).

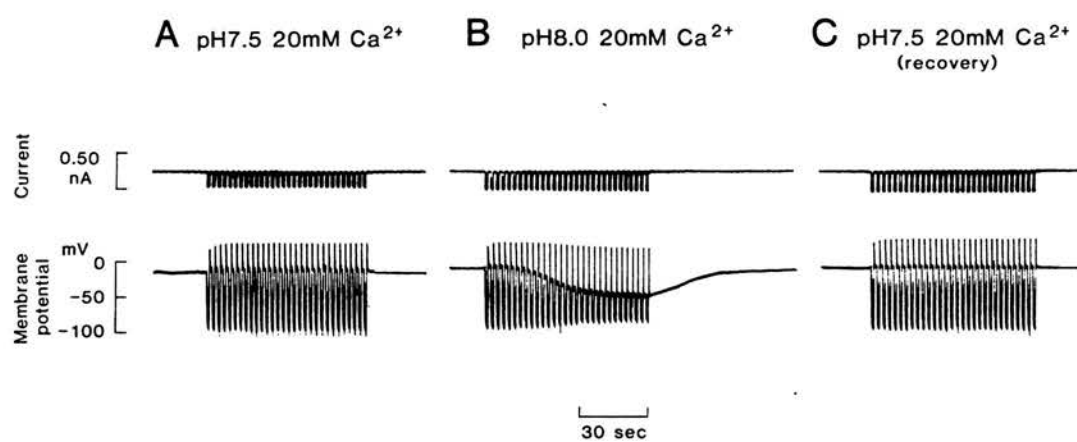


Figure 6.7. The effect of pH 8.0 on calcium-loading of the egg via action potentials. A, anode-break responses at pH 7.5, B, the same frequency of anode-break responses evokes a hyperpolarisation at pH_o 8.5. C, the recovery of the membrane at pH_o 7.5; no hyperpolarisation occurs. Lower trace, membrane potential; upper trace, current.

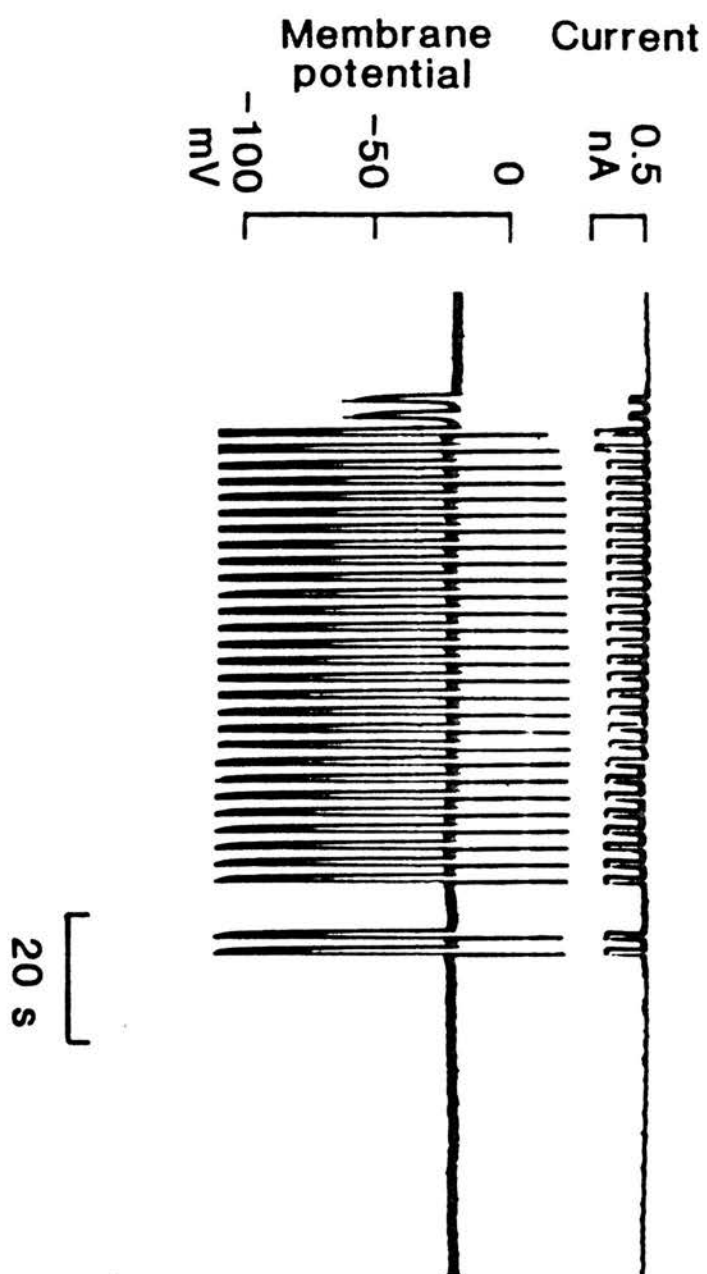


Fig 6.8 Suppression of the delayed hyperpolarisation induced by a single action potential; a series of calcium action potentials evoked by anode-break responses fail to evoke a hyperpolarisation in an EGTA-loaded egg. Upper trace, current; lower trace, membrane potential.

E Is the lack of a second response to an action potential due to desensitisation of the Ca^{2+} -activated K^+ channel ?

The absence of a second large response to an action potential might have been due to a change in the sensitivity of the Ca^{2+} -activated K^+ channels after this initial rise in $[\text{Ca}^{2+}]_i$. This was investigated by injecting sufficient Ca^{2+} from a Ca^{2+} microelectrode to activate these channels directly (5-6 nC); no decrease in the size of each hyperpolarisation was observed on repetitive injection (Fig.6.9 A). This large current was chosen to avoid complications regarding the refractory period of the response reported by Igusa & Miyazaki (1983). They used a maximum injection current of 2 nA; with this current size it was possible to demonstrate a refractory period for the large all-or-none response evoked in the hamster egg.

Thus, the intracellular Ca^{2+} stores probably influenced the size of the Ca^{2+} -evoked hyperpolarisations they observed. At a higher pH_o (8.5), a slight facilitation could be detected during repeated injection and the responses were markedly prolonged (Fig.6.9 B), a feature discussed in Section 4. Thus, at neither pH value was a decrease in the size of the hyperpolarisation seen with consecutive Ca^{2+} injections of the same size.

F Various treatments of the egg suppress a transient hyperpolarisation in response to a single action potential.

During the Ca^{2+} injection experiments it was observed that the insertion of a Ca^{2+} microelectrode caused a transient membrane hyperpolarisation (Igusa & Miyazaki, 1983) and thereafter a delayed transient response could not be evoked by Ca^{2+} influx (Fig.6.10). Once again, the responses became graded with the number of stimuli (Fig.6.10 B,C and D). The reversal potential for the response produced by insertion of the Ca^{2+} pipette was -71.3 ± 2.8 mV ($n=3$), somewhat more positive than the equilibrium potential for K^+ . The discrepancy is probably due to the leakage pathway around the pipette (Georgiou et al, 1987a).

The capacity of the cell to produce a delayed large hyperpolarisation to a single action potential could be impaired by treatments other than insertion of a Ca^{2+} electrode. All of these treatments caused large transient hyperpolarisations. The application of solutions with high pH (in the range 8.0 - 9.5) for example, produced such a response and thereafter a single action potential failed to evoke a delayed hyperpolarisation (see C, Section 6). Furthermore, application of 1 mM La^{3+} to the bath produced one or two transient hyperpolarisations ($n=3$); La^{3+} is a known inhibitor of the Ca^{2+} pump (Sarkadi, Szasz, Gerlocozy & Gardos, 1977; see also Georgiou et al, 1987). Likewise, bathing the cell in a Na^+ -free solution produced one or two transient hyperpolarisations ($n=3$), resulting from inhibition of the Na^+ - Ca^{2+} exchange (see Section 5). After these treatments, both of which produce transient rises in $[\text{Ca}^{2+}]_i$, a single action potential was unable to evoke a delayed hyperpolarisation in these eggs.

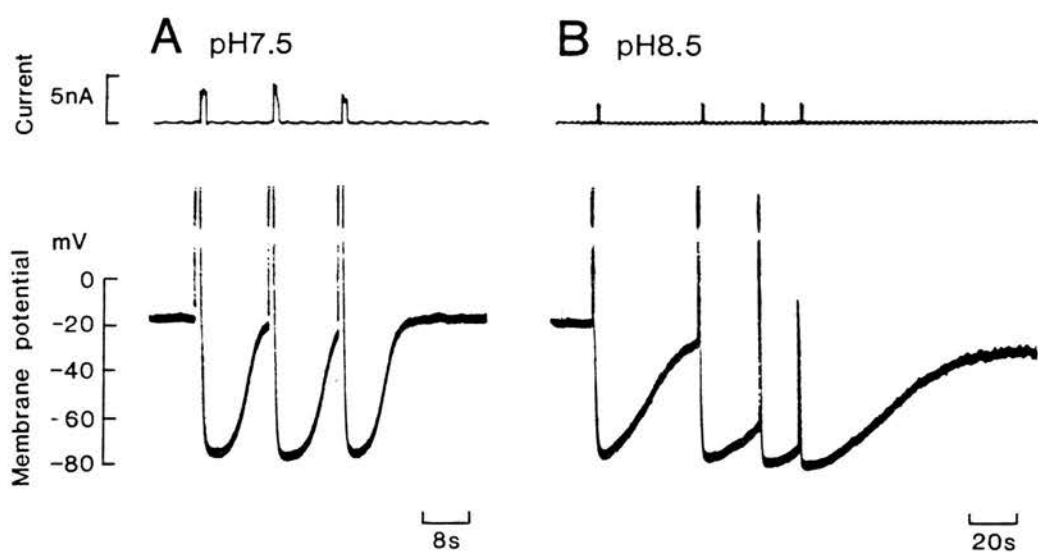
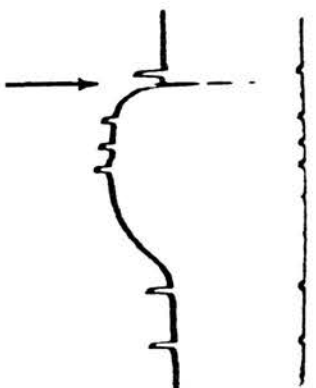


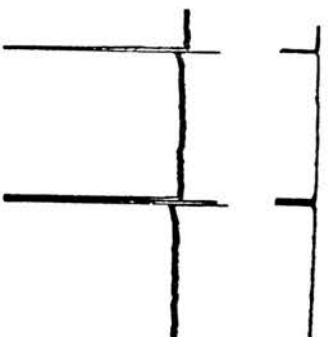
Figure 6.9. Repetitive calcium injections show that at both pH_o 7.5 (**A**) and 8.5 (**B**), there was no evidence of a refractory period of the calcium-activated potassium channel (i.e. that the channel itself did not become desensitised) the responses did not decrease in amplitude or duration.

Current
0.5
nA
Membrane
potential
-50
-100
mV

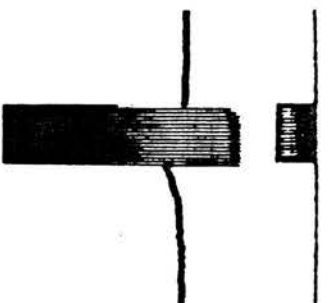
A



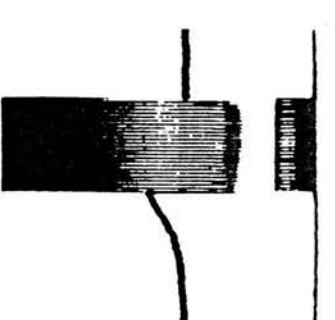
B



C



D



20 s

Figure 6.10. Insertion of a second electrode containing 1 M calcium chloride in to a hamster egg (at the arrow in **A**) produced a hyperpolarisation accompanied by an increase in conductance (monitored by the six electrotonic potentials present on the trace). Subsequent anode break responses produce graded hyperpolarisations (^{B, C & D}~~C, D & E~~), increasing in size with the number of stimuli. Upper trace, current; lower trace, membrane potential.

G Calcium injections suggest a possible involvement of intracellular Ca^{2+} stores in the response to an action potential.

The latency between Ca^{2+} influx during the action potential and the transient response described above in (C) suggests the activation of a mechanism which causes a large delayed rise in $[\text{Ca}^{2+}]_i$. Igusa and Miyazaki (1983) proposed Ca^{2+} -induced Ca^{2+} release from intracellular stores as the mechanism responsible for the generation of the transient responses recorded at fertilisation. They based their proposal on two key findings, namely a) the existence of a critical Ca^{2+} injection current above which the response suddenly became much larger and which is referred to as the regenerative response by Igusa and Miyazaki and b) the presence of a refractory period after the induction of a regenerative response. In performing Ca^{2+} injections at 26 °C, only one 'all-or-none' response could be evoked by them.

Attempts to elicit a large transient response with 'all-or-none' properties by intracellular iontophoretic injection of Ca^{2+} proved unsuccessful in all (n=10) but one experiment at room temperature in this study. Typically the effect of increasing the strength of the Ca^{2+} current pulse was a progressive increase in both the amplitude and the duration of the response as illustrated in Fig.6.11 A. The area under a response (V_m) was chosen by Kuba (1980) and Igusa and Miyazaki (1983) as a parameter because it took account of changes in both the amplitude and duration of a response. The relation between the quantity of the injected Ca^{2+} and the area under the responses shown in Fig.6.11 B (open circles) showed no apparent discontinuity and could be adequately described by a sigmoid curve. The results of a similar experiment are shown in Fig.6.11 B (closed circles). Again the data could be fitted with a sigmoid curve.

Since Igusa and Miyazaki (1983) also plotted the rise in K^+ conductance g_K against the quantity of injected Ca^{2+} , the variation of these two parameters with the amount of injected Ca^{2+} for identical responses was plotted in order to exclude the possibility that they are described by different mathematical relations. As is evident from Fig.6.11 C, both g_K and the area under a response varied in a similar fashion with the quantity of injected Ca^{2+} .

In order to test further the likely involvement of Ca^{2+} -induced Ca^{2+} release in the action potential-induced hyperpolarisation, caffeine was applied extracellularly at a concentration of 10-20 mM. Caffeine is known to promote a release of Ca^{2+} by augmenting the sensitivity of the Ca^{2+} -induced Ca^{2+} release in the sarcoplasmic reticulum of skeletal muscle (Endo, 1977; Dux and Martonosi, 1984). Caffeine when applied to hamster eggs at pH 7.5 for periods exceeding 30 min failed to induce a hyperpolarisations (n=6).

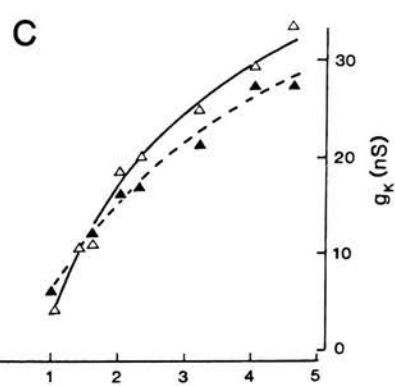
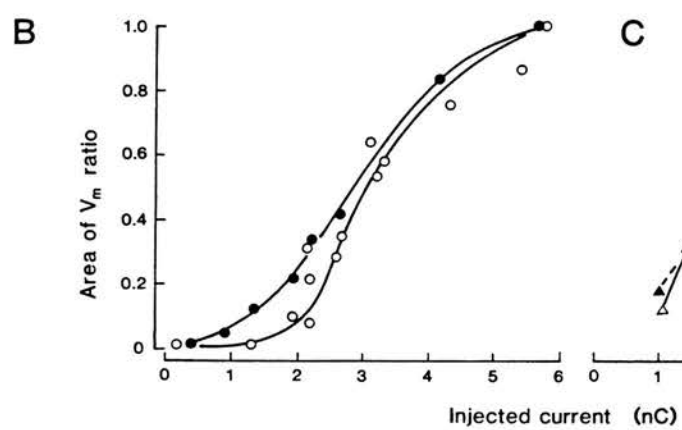


Figure 6.11. Investigation of the effect of calcium injections of different sizes. **A**, Increasing the size of the injection current produced a larger hyperpolarisation. **B**, a sigmoid curve was obtained when the size of injected current was plotted against the area under the response. The closed circles and closed triangles are values taken from the same cell, the open circles and triangles are from another cell.

Discussion.

A transient all-or-none K^+ conductance^{was} evoked after a long latency by a single Ca^{2+} action potential in some unfertilised hamster eggs. Previous studies had suggested a contribution of Ca^{2+} release from intracellular stores to hyperpolarisations in hamster eggs (Igusa & Miyazaki, 1983; Miyazaki, 1987a). This study provides further evidence, showing that a large hyperpolarisation with a delay can be evoked by a single Ca^{2+} action potential. A second response of comparable size to the first delayed transient response (Fig.6.3) was difficult to induce by a single action potential.

This observation agrees with the result obtained by Ca^{2+} -injection (Igusa & Miyazaki, 1983); they say that once a full response occurred, a second response could not be induced at 26 °C. It is probable that at the temperature we used (22-24 °C), the intracellular Ca^{2+} pumping system is less efficient, and it takes more time to replenish the internal stores until the threshold concentration triggering Ca^{2+} release from intracellular stores. Potentially the Ca^{2+} influx during an action potential might be sufficient to rapidly induce opening of small numbers of Ca^{2+} -activated K^+ channels (as suggested by the undershoot following action potentials in Fig.1, insert). However, the large responses reported here occur after a long delay (ca. 9 s) and seem to indicate the presence of a process which can amplify the expected small rise in $[Ca^{2+}]_i$ following an action potential. For example, the rise in $[Ca^{2+}]_i$ might trigger Ca^{2+} release from a store as proposed by Igusa & Miyazaki (1983).

Recently, Miyazaki (1987) showed that injection of IP_3 produced a rise in $[Ca^{2+}]_i$ in the hamster egg. Influx of Ca^{2+} via an action potential might promote the production of a second messenger, causing Ca^{2+} release from a store, as suggested by Whitaker & Aitchison (1985) for sea urchin eggs.

However recent studies in this preparation show that the Ca^{2+} influx probably occurs after IP_3 production (Irvine & Moor, 1986). They propose that IP_3 elicits full activation of sea urchin eggs firstly, by releasing Ca^{2+} from endoplasmic reticulum and secondly, by being converted to IP_4 , which opens plasma membrane Ca^{2+} channels. This would allow for the possibility that second messengers directly activate non-voltage gated Ca^{2+} channels in the hamster egg and thus augment Ca^{2+} influx, as observed with IP_3 in T-lymphocytes (Kuno & Gardner, 1987). Yet another possibility is that a second messenger (IP_4) blocks Ca^{2+} extrusion (Popescu, Hinescu, Musat, Ionescu & Pistritzu, 1986) and hence causes a rise in $[Ca^{2+}]_i$ by virtue of a maintained or elevated Ca^{2+} influx.

Suppression of a second all-or-none response to an action potential^{occurred}. In trying to inject small amounts of Ca^{2+} into the egg to mimic the Ca^{2+} entry through voltage-gated Ca^{2+} channels, a technical difficulty arose. When the Ca^{2+} pipette was inserted into the egg, the resulting rise in $[\text{Ca}^{2+}]_i$ caused a large hyperpolarisation and presumably the slow amplification system was again stimulated because the sensitivity to a single action potential was removed (Fig.6.10).

High pH_o is known to inhibit Ca^{2+} extrusion from hamster eggs (Georgiou, House, McNiven & Yoshida, 1988) leading to a rise in $[\text{Ca}^{2+}]_i$. In eggs at pH 8.0, the passive influx of Ca^{2+} was often sufficient to trigger the slow amplification system. Other conditions including the application of sodium-free solution (blocks the Na^+ - Ca^{2+} exchange; Reuter and Seitz, 1968) or solutions containing La^{3+} (blocks the Ca^{2+} pump; Sarkadi et al, 1977) were also effective in producing a hyperpolarisation.

There are several possibilities why a second delayed transient hyperpolarisation could not be obtained by an individual action potential: 1) partial depletion of the Ca^{2+} store resulting from impaired re-uptake, 2) desensitisation of the Ca^{2+} store to further stimulation, 3) interference in the production of a second messenger or 4) inactivation of non-voltage gated Ca channels.

Iontophoretic Ca^{2+} injection provides further evidence for involvement of an intracellular Ca^{2+} store. It would be expected that if intracellular Ca^{2+} stores were involved in the activation of the large, delayed transient response to a single action potential, then a non-linearity between response and Ca^{2+} injection current would be seen. The sigmoid relation between response and Ca^{2+} injection current (Fig.6.11 B) is consistent with the operation of such an amplification system; it can be interpreted as a form of cooperativity. Indeed, Igusa and Miyazaki (1983) proposed that the occasional discontinuity observed in their stimulus response curves was caused by Ca^{2+} -induced Ca^{2+} release from internal stores. In this study, such discontinuities were not observed as frequently; the temperatures used here were sufficiently lower than those used by Igusa and Miyazaki to account for this. Additionally, Igusa and Miyazaki did not use a virtual ground system (Section 2) to measure Ca^{2+} injection current (personal communication from Dr. S.Miyazaki); the discontinuities they observed may result from miscalculation of injected current. The possibility cannot be excluded, however, that the non-linear behaviour (Fig.6.11 B) reflects a non-linear dependence of g_K on $[\text{Ca}^{2+}]_i$ (Barrett, et al, 1982).

The failure of caffeine to evoke a hyperpolarisation does not eliminate a role for intracellular Ca^{2+} stores in the hyperpolarisation; it may have been due to an insensitivity of the intracellular stores of hamster egg to this drug.

The evidence presented in this Section indicates that a small rise in $[\text{Ca}^{2+}]_i$, due to an action potential, activates a slow process leading to a further large increase in $[\text{Ca}^{2+}]_i$. At fertilisation a

rise in $[Ca^{2+}]_i$ occurs, and perhaps an amplification system similar to that discussed here underlies the large transient hyperpolarisations recorded.

SECTION 7 Fertilisation of hamster eggs.

Fertilisation of hamster eggs results in a series of transient hyperpolarisations of the egg plasma membrane (Miyazaki and Igusa, 1981). These hyperpolarisations are due to an increase in K^+ conductance (Miyazaki & Igusa, 1982) mediated by a rise in the intracellular free Ca^{2+} concentration (Igusa and Miyazaki, 1986). These repetitive hyperpolarisations are superimposed on a slow hyperpolarising shift of the membrane potential of about 20 mV from the resting potential.. Both the repetitive hyperpolarisations and the hyperpolarising shift are inhibited by 9 mM Mn^{2+} and 9 mM Co^{2+} (Igusa and Miyazaki, 1983).

The results presented in this Section form an investigation of the effects of some Ca^{2+} channel blockers and inhibitors of Ca^{2+} extrusion on the activation of Ca^{2+} -activated K^+ channels by sperm. The characteristics of the hamster fertilisation potential and the effects of these chemicals on the fertilisation potential are discussed.

Results.

A. Characteristics of fertilised eggs

None of the unfertilised eggs examined in the course of this study displayed either single or repetitive spontaneous hyperpolarisations. The resting potential and input resistance of ten representative eggs used in these experiments were -26.6 ± 4.3 mV and 400 ± 134 M Ω respectively. In the hamster, the major block to polyspermy resides in the zona pellucida; addition of sperm to the zona-free hamster egg usually resulted in severe polyspermy in these experiments.

A typical fertilisation potential resulting from insemination of a hamster egg is illustrated in Fig.1. Addition of the sperm-containing solution (capacitiation medium, see Section 2) to the bath containing the egg immediately produced a 5 - 10 mV depolarisation of the membrane which recovered after about 10 s (first arrow, Fig.7.1). This small depolarisation was not sperm-induced as it could be evoked by sperm-free solution. After testing the various components of the capacitating medium, adrenaline was found to be the cause of this small depolarisation.

Current

0.15nA

Membrane Potential

0
-20
-40
-60
-80
mV

1st at. | 2nd at. | 3rd at.

1st st.

2nd st.

sperm added

1min

Figure 7.1. Intracellular recording of the fertilisation of a zona-free hamster egg, showing repetitive hyperpolarisations with accompanying decrease in input resistance. Upper trace, current; lower trace, membrane potential. Sperm-containing solution was added at the arrow, producing a small depolarisation. The time that the first individual sperm attaches to the egg is noted at the arrow near '1st at.', the second sperm to attach by '2nd at.' etc. The time that the first sperm stops moving and has a straightened tail is noted at '1st st.', the second by '2nd st.'

The first electrical event observed due to contact of a sperm with the egg at its resting potential was one of a series of transient hyperpolarisations. The first two to four of these hyperpolarisations had a similar amplitude and duration (31.5 ± 5.3 mV and 6.9 ± 2.9 s, respectively, $n=32$). These values were obtained from 10 representative eggs. The membrane potential and input resistance at the peak of these hyperpolarisations was -60.0 ± 6.4 mV and 154.1 ± 67.9 M Ω , respectively ($n=32$).

Subsequent hyperpolarisations were slightly decreased in amplitude and increased in duration, but continued until the end of most experiments (at least 1 hour). The interval between hyperpolarisations varied from egg to egg (20 to 60 s), but was generally constant for each egg after the first three hyperpolarisations.

In an attempt to quantify the interval between hyperpolarising responses, the number of hyperpolarisations in a given time was counted and then the frequency of hyperpolarising responses was expressed as an average that would occur in 5 min, that is, X number of HR/5 min, where HR stands for hyperpolarising response (Igusa and Miyazaki, 1983). The hyperpolarisations in these eggs had an estimated reversal potential (see Section 2) of -84.0 ± 5.7 mV ($n=32$), similar to the previously reported values (Miyazaki and Igusa, 1981).

After sperm attachment, the flagellar activity decreased dramatically and usually within two minutes, one sperm stopped moving and became straight. Sperm straightening will be indicated in the figures, where relevant, by 'st.'. A reasonable correlation between this straightening and the first hyperpolarising response has been demonstrated by Miyazaki and Igusa (1981). As Miyazaki and Igusa (1981) reported, a few eggs responded to sperm addition with only two or three hyperpolarisations and then became silent.

Hyperpolarisations did not summate, but appeared in an all-or-none manner. The repetitive hyperpolarisations were independent of sperm attachment after they had been initiated in the egg by the first sperm; occasionally, only one sperm would attach and repetitive hyperpolarising responses would continue. Confirmation of fertilisation was occasionally made by observation of male pronuclei or decondensing sperm heads in the egg: Miyazaki and Igusa (1981) have demonstrated that repetitive hyperpolarising responses are themselves a reliable indication of fertilisation.

B The dependence of the hyperpolarising responses on $[Ca^{2+}]_o$ and the inhibitory effects of various Ca^{2+} channel blockers.

Replacement of Ca^{2+} by Mg^{2+} or Ba^{2+} abolishes the hyperpolarising responses. The dependence of the hyperpolarising responses on the external Ca^{2+} concentration was examined by replacing the external Ca^{2+} with Mg^{2+} (12 mM).

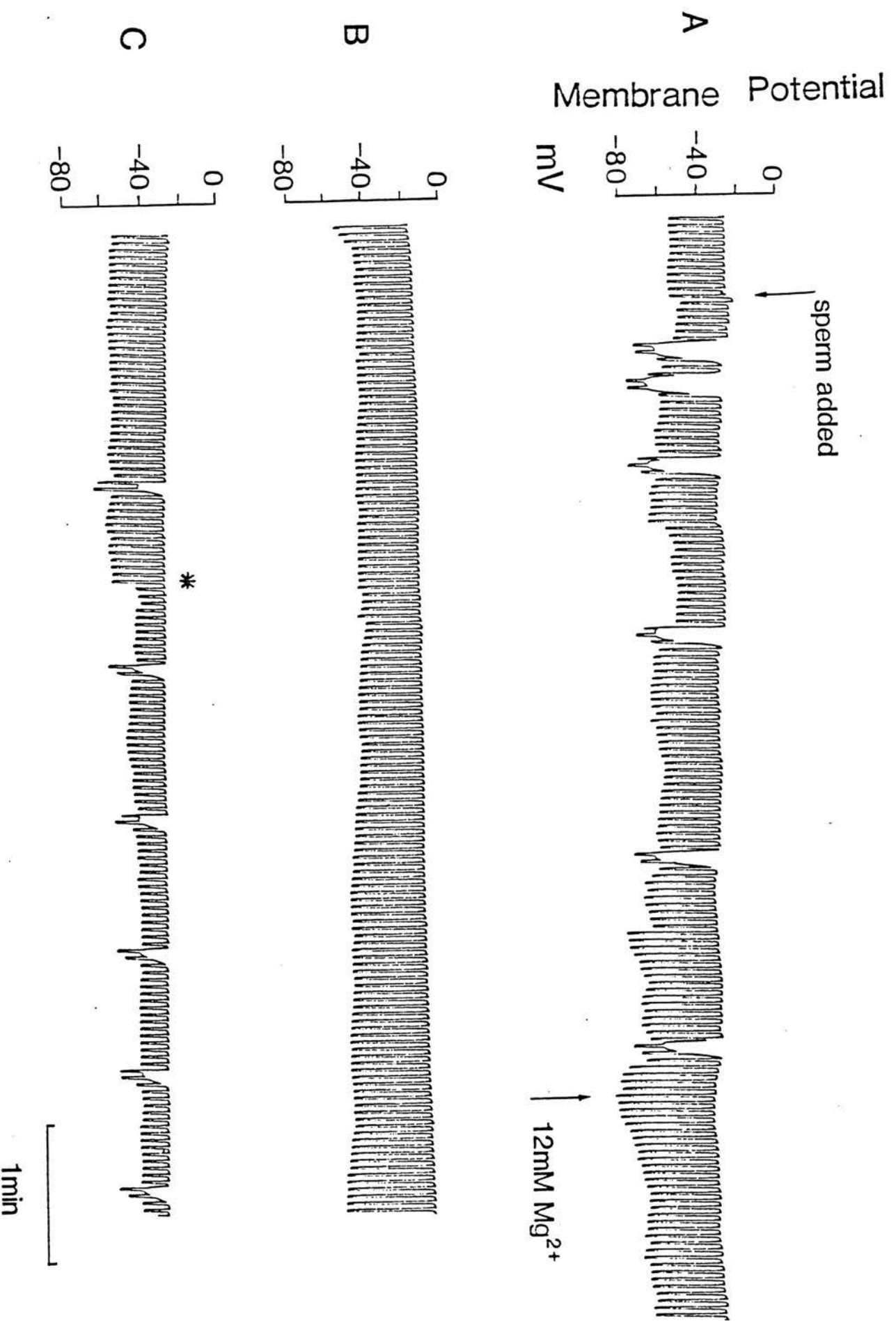


Figure 7.2. Abolition of hyperpolarising responses by replacement of calcium with magnesium.

A, sperm added to the solution at the time indicated by the first arrow produced six hyperpolarisations in the egg before perfusion with magnesium solution (second arrow). Magnesium solution led to a small depolarisation of the membrane potential. B, hyperpolarisations were abolished in 12 mM magnesium. Recovery of hyperpolarising responses occurred after washing with 4 mM calcium solution (not shown). C, the frequency of responses in 10 mM calcium solution was greater than that in normal solution. The size of the constant-current pulses applied to produce the brief hyperpolarising deflexions (electrotonic potentials) of the membrane potential (electrotonic potentials) was reduced at the position indicated by the asterisk.

To give an idea of the frequency of occurrence of the hyperpolarisations in normal solution, several were recorded after the sperm was added (in the case of Fig.7.2, six were recorded, giving a frequency of an average of 4.3 HR/5 min). Perfusion of the egg with Ca^{2+} -free solution resulted in the abolition of the hyperpolarising responses and the hyperpolarising shift (Fig.7.2 B). The stabilising action of 12 mM Mg^{2+} was not quite sufficient to maintain the membrane potential at the same level as that seen with 4 mM Ca^{2+} ; a 10 mV depolarisation occurred.

The hyperpolarising responses resumed after washing the egg with 4 mM Ca^{2+} . By raising the $[\text{Ca}^{2+}]_o$ to 10 mM, the frequency of the hyperpolarisations (6.2 HR/5 min) was increased compared to the frequency obtained at 4 mM Ca^{2+} concentration (Fig.7.2 C). Additionally, the amplitude of the hyperpolarisations was decreased at this higher concentration of $[\text{Ca}^{2+}]_o$, from 34.5 ± 3.4 mV in 4 mM ($n=5$) to 14.6 ± 1.3 mV in 10 mM Ca^{2+} ($n=5$). The shift in the resting potential to the hyperpolarising direction due to 10 mM Ca^{2+} was not sufficient to be responsible for the decrease in the amplitude of the hyperpolarisations.

A similar effect was obtained with replacement of Ca^{2+} by 4 mM Ba^{2+} ; the hyperpolarising responses and the hyperpolarising shift were abolished. Perfusion with normal solution recovered the hyperpolarisations, but 5 min of perfusion with 4 mM Ca^{2+} was required to re-initiate the hyperpolarising shift. Once again, the frequency of the hyperpolarising responses in 10 mM compared to 4 mM Ca^{2+} was increased from 6 HR/5 min to 7 HR/5 min ($n=3$) and their amplitude decreased from 29.8 ± 1.7 mV to 11.5 ± 0.7 mV ($n=3$).

Inhibition of the hyperpolarising responses by cadmium or cobalt.

In an attempt to obtain information on the Ca^{2+} channels responsible for influx of Ca^{2+} into the egg during fertilisation, different Ca^{2+} channel blockers were used. The presence of the Ca^{2+} channel blocker Cd^{2+} (0.2 mM) produced a decrease in the frequency of the hyperpolarisations (from 6.9 to 4.0 HR/5 min) and completely abolished the hyperpolarising shift ($n=3$).

Another effect of Cd^{2+} was to produce a gradual decrease in the amplitude of the hyperpolarisations, from 28.1 ± 5.8 mV in normal solution to about 10 mV by the fifth hyperpolarisation after cadmium application. These phenomena are illustrated in Fig.7.3. No change in the reversal potential of the hyperpolarisations was seen after the Ca^{2+} channel blocker was added; E_R was -84.2 ± 4.4 mV in normal solution and -85.1 ± 14.3 mV in the presence of Cd^{2+} ($n=4$).

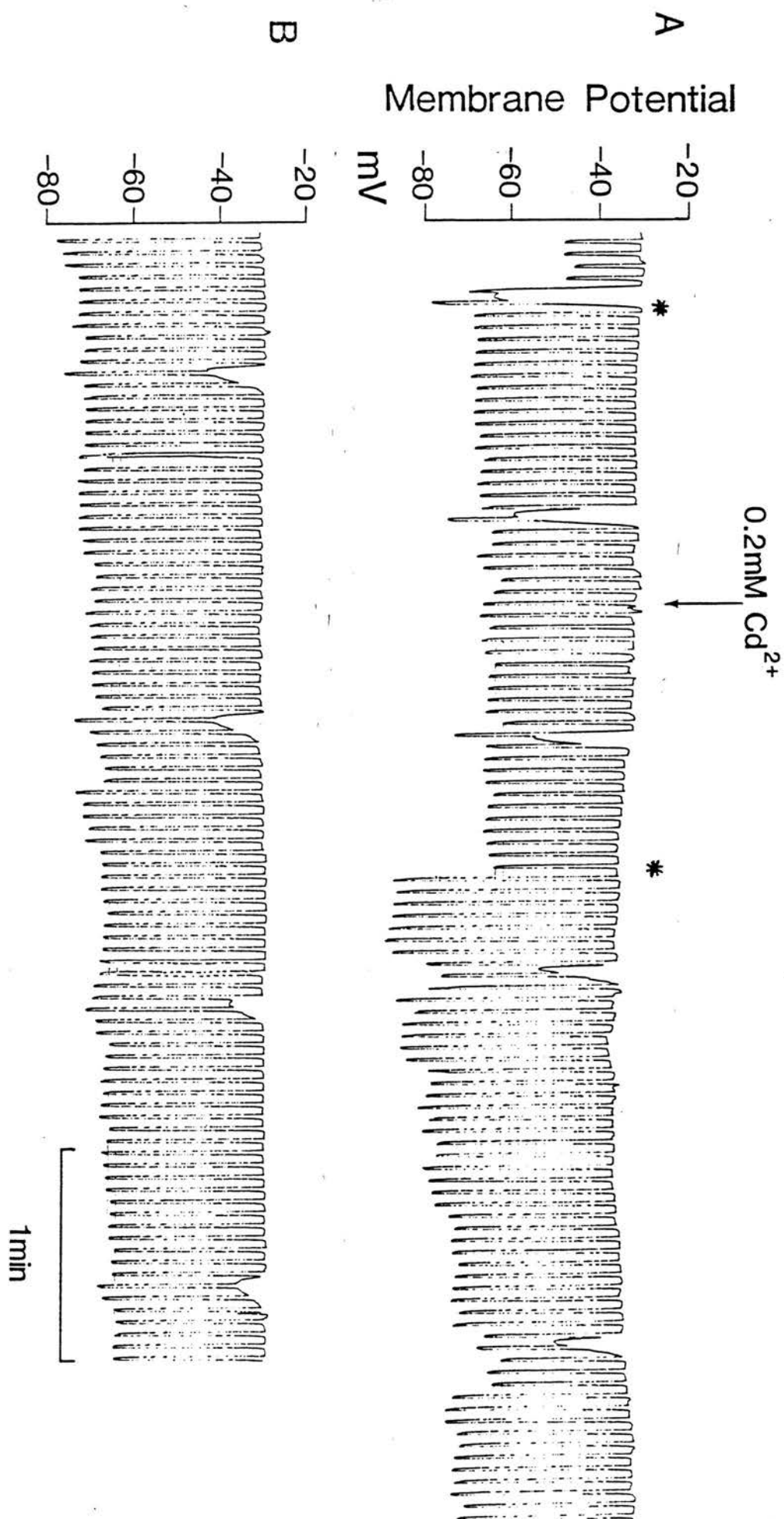
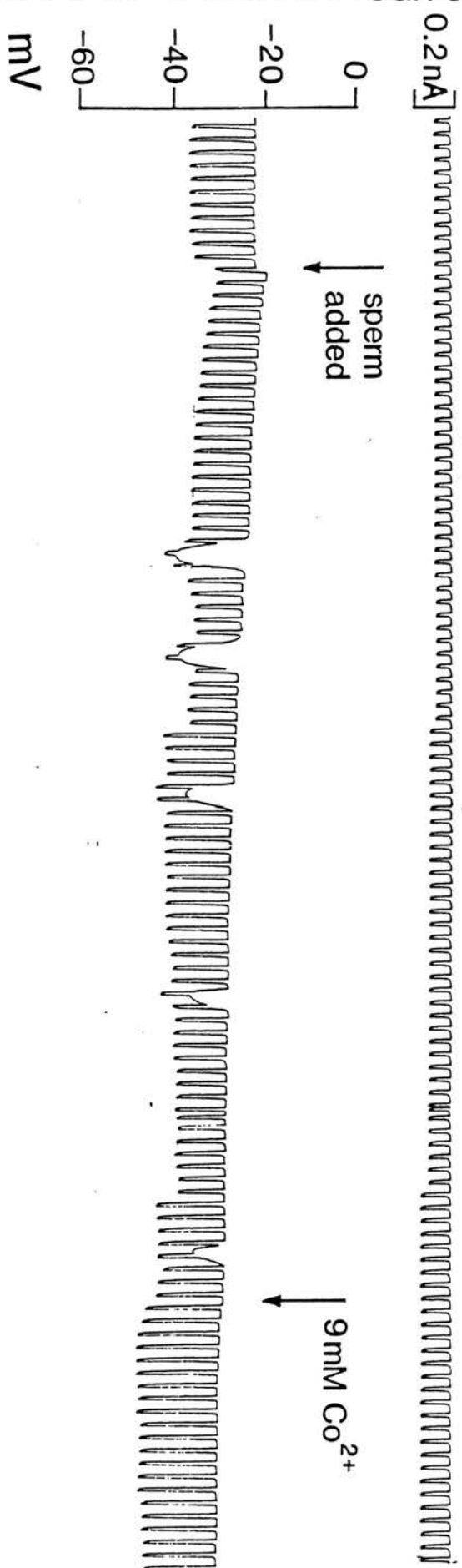


Figure 7.3. The amplitude and frequency of the hyperpolarising responses was decreased when the calcium channel blocker cadmium was added to the bathing solution (at the arrow in A). Figure B is a continuation of Figure A. The size of the constant-current pulses applied to produce the brief hyperpolarising deflexions of the membrane potential was increased at the positions indicated by the asterisks.

Membrane Potential Current

A



B

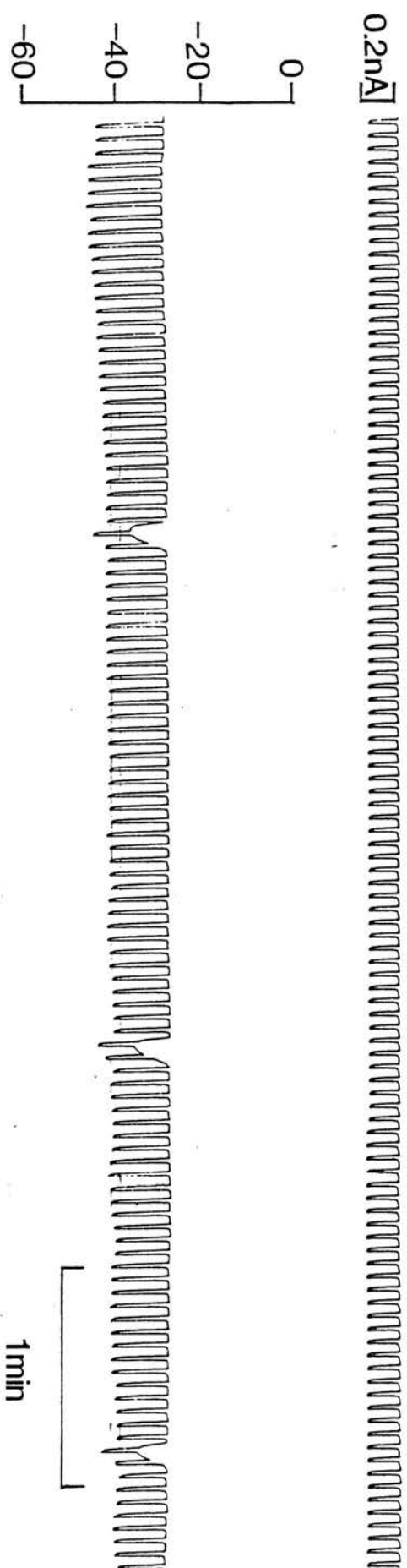


Figure 7.4. Addition of 9 mM cobalt to the solution bathing the egg decreased the frequency of the hyperpolarising responses (at second arrow). Little effect on the amplitude of the hyperpolarisations was seen. Figure B is a continuation of Figure A. Upper trace, current; lower trace, membrane potential.

The results obtained with the Ca^{2+} channel blocker cobalt were slightly different. For a given concentration of Co^{2+} , the frequency of the hyperpolarisations was decreased, but the amplitude was almost unaltered; in Fig.7.4, for example, 9 mM cobalt was used. With this concentration of cobalt, the frequency of the hyperpolarisations decreased from 5.5 HR/5 min in normal solution to 2.4 HR/5 min in the presence of Co^{2+} . The hyperpolarising shift was again halted by the presence of this Ca^{2+} channel blocker.

Estimates of the reversal potential for these responses showed that they were affected by cobalt; E_R was -76.8 ± 5 mV in the absence of, and -87.0 ± 1 mV in the presence of cobalt ($n=5$). This may be due to surface potential effects of this high divalent cation concentration. Raising the cobalt concentration to 12 mM was sufficient to abolish the hyperpolarising responses until washing with normal solution (Fig.7.5). The membrane potential on this trace (Fig.7.5B) exhibited some small, periodic depolarisations associated with an increase in input conductance.

It is possible that the surface potential effects of Co^{2+} are such that membrane potential changes associated with periodic rises in $[\text{Ca}^{2+}]_i$ are masked. However, this effect was only seen in this egg; the others did not display this phenomenon.

The greater sensitivity of the hyperpolarising shift, compared with the hyperpolarising responses, to these Ca^{2+} channel blockers may suggest that the hyperpolarising shift is not completely due to Ca^{2+} -activated K^+ channels, as proposed by Igusa and Miyazaki (1983)

C The dual effects of lanthanum, a Ca^{2+} channel blocker and an inhibitor of Ca^{2+} extrusion, on the fertilisation potential.

Addition of lanthanum to the bath before addition of the sperm prevented any hyperpolarising responses from occurring ($n=6$). These experiments were performed on days when the success rate of fertilisation was otherwise 100 %, making it unlikely that the lack of response was due to unresponsive eggs. However, as this result might have been due to effects of lanthanum on the sperm themselves, rather than because of an inhibition of Ca^{2+} influx, no definite conclusions are drawn from this result.

Like the Ca^{2+} channel blockers above, lanthanum produced a decrease in the frequency of the hyperpolarising responses.

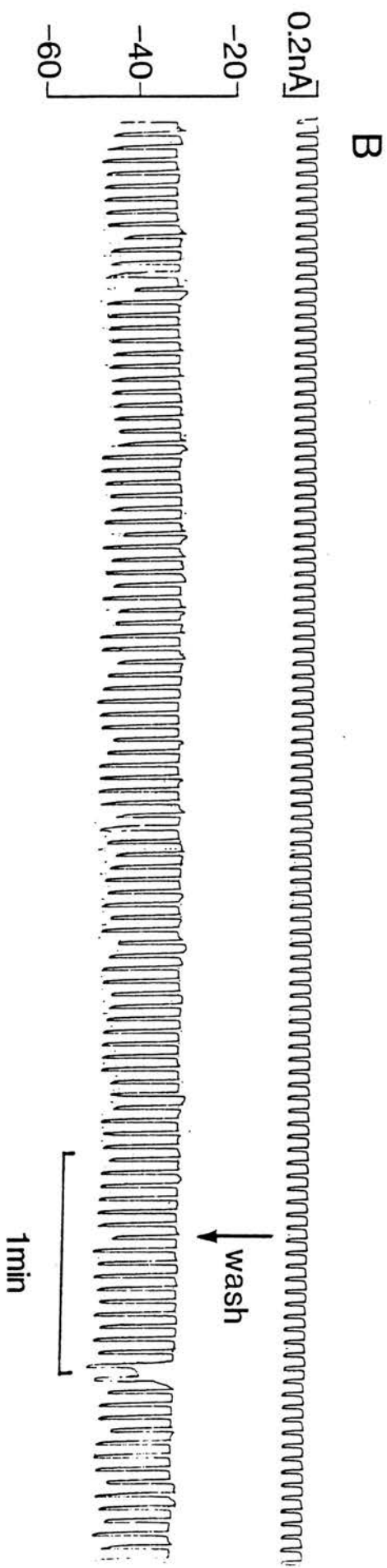
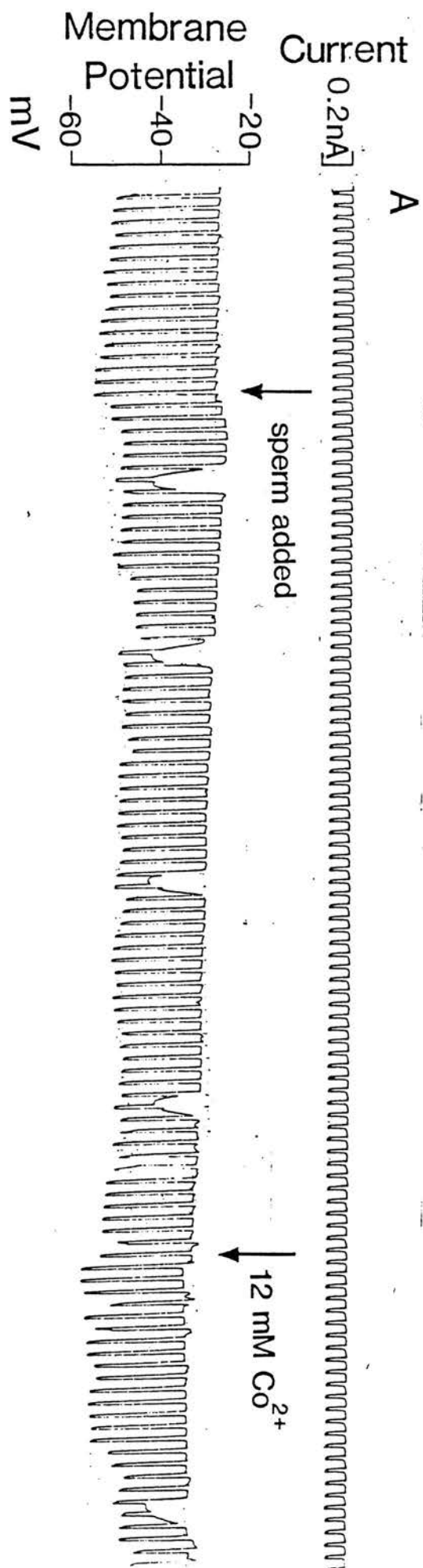


Figure 7.5. **A**, Addition of 12 mM cobalt (first arrow) to the solution bathing the egg abolishes the hyperpolarising responses induced by sperm addition (second arrow). The hyperpolarising shift is also abolished. **B** shows recovery of hyperpolarising responses after washing with normal solution. Also present are small depolarisations of the membrane potential induced by cobalt. In **A** and **B**, the upper trace shows current, the lower trace shows membrane potential.

Membrane Potential

Current

-20
-40
-60
mV

0.2 nA

100 μ M La^{3+}

1 min

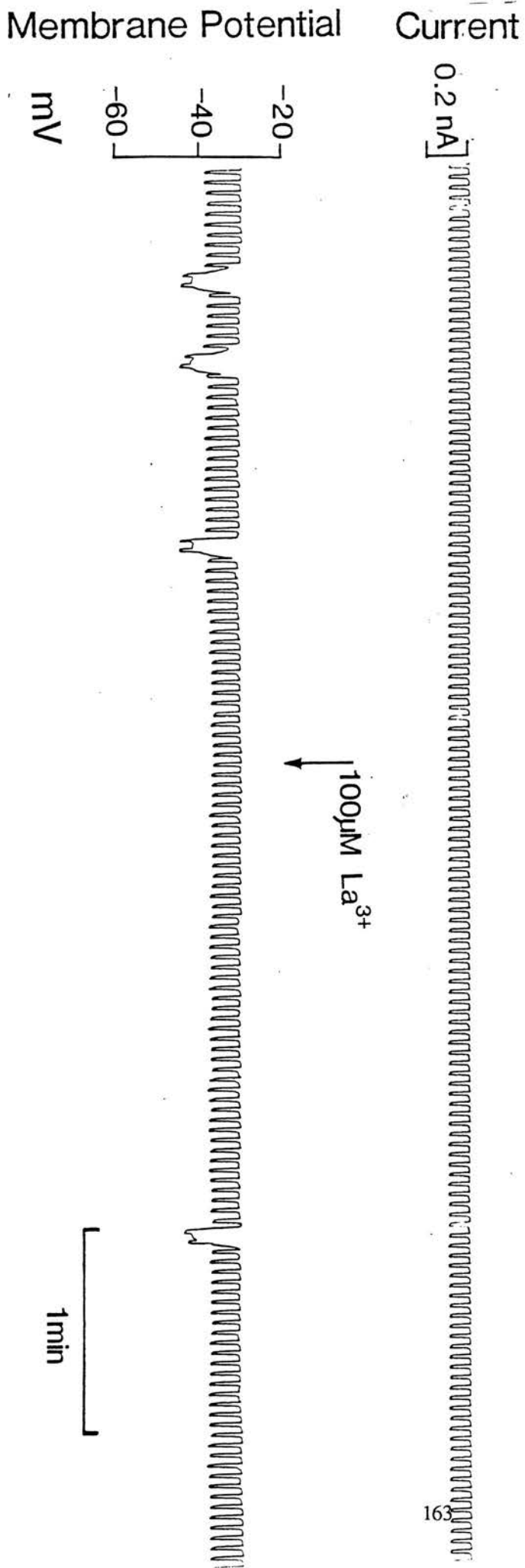


Figure 7.6. Addition of 0.1 mM lanthanum (at the time indicated by the arrow) to the solution bathing the egg decreases the frequency of the hyperpolarising responses with little change in the characteristics of the responses (either amplitude, duration or reversal potential). Upper trace, current; lower trace, membrane potential.

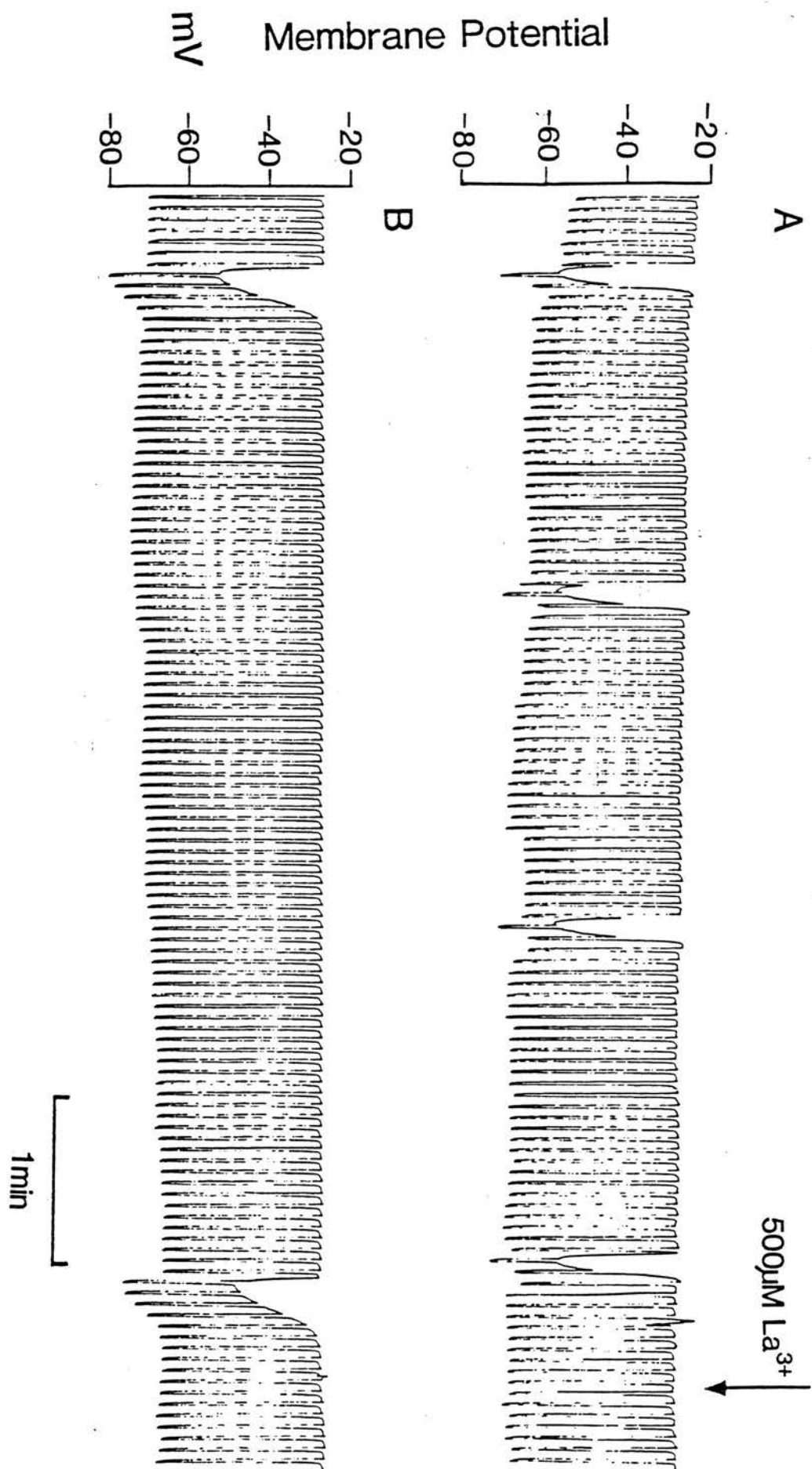


Figure 7.7. Increasing the concentration of lanthanum added to the solution produced an increase in the duration of the responses as well as a decline in their frequency. **A**, addition of 0.5 mM lanthanum was added to the bathing solution at the time indicated by the arrow. **B**, reduction in frequency and prolongation of responses by lanthanum. The recovery of duration and frequency occurred after washing with normal solution (not shown).

The results obtained with lanthanum were concentration-dependent; at a concentration of 100 μM , the decrease in frequency was not accompanied by changes in the size or time course of the hyperpolarising responses (Fig.7.6). The decrease in frequency caused by 100 μM lanthanum was from 5 HR/5 min (in normal solution) to 2.3 HR/5 min. This was a similar result to that obtained with cobalt. Again, the hyperpolarising shift was halted. When the concentration was raised to 500 μM (Fig.7.7), in addition to the decrease in frequency of the hyperpolarising responses, the duration of these responses was increased from 6.0 ± 1.3 s ($n=6$) to 10.0 ± 2.2 s ($n=4$). The durations of the hyperpolarisations were calculated from $T_{1/2(V)}$, the duration of the response at half its amplitude (see Section 2).

The increase in input resistance and hyperpolarisation of the membrane potential observed at these concentrations of lanthanum is discussed in Section 3 (effect of La^{3+} on the passive electrical properties of the hamster egg). Addition of 1mM lanthanum to the egg also produced a larger increase in the duration of the hyperpolarising responses from 6.7 ± 1.0 s in normal solution ($n=5$), to 12.9 ± 2.3 s in the presence of 1 mM lanthanum ($n=4$).

The estimated reversal potential of these hyperpolarisations in the presence of La^{3+} was -83.2 ± 6.1 mV ($n=12$), similar to that obtained in normal solution. The effects of La^{3+} were reversible; hyperpolarising responses with similar characteristics to those before La^{3+} addition were obtained by perfusing the egg with normal solution. Also, the hyperpolarising shift was re-initiated after perfusion with normal solution.

D Fertilisation of the hamster egg in sodium-free solution.

Replacement of sodium by choline chloride had an effect on the passive electrical properties of the hamster egg; it produced a hyperpolarisation of the membrane potential and an increase in input resistance compared to normal solution (to -37.2 ± 2.8 mV and 485 ± 74.2 M Ω , respectively). These effects are compatible with removal of a sodium conductance contributing to the resting potential.

Insemination of hamster eggs in a Na^+ -free solution produced a fertilisation potential similar to that obtained in normal solution. In Fig.7.8 is an example of an egg fertilised in sodium-free solution. The repetitive hyperpolarisations could be made to reverse to depolarisations by injection of steady d.c. current, shifting the egg to potentials more hyperpolarised than the equilibrium potential for K^+ (Fig.7.8 B). At a potential of -80 mV (near the equilibrium potential for K^+), the membrane potential was not greatly displaced, but the rise in conductance was still

observed (Fig.7.8C). The hyperpolarisations were also recorded at - 20 mV (Fig.7.8D) and the resting potential of -40 mV (Fig.7.8A) . As this is not a voltage-clamp experiment, comparison between the size of the hyperpolarisations cannot be made.

Membrane Potential Current

0.2nA]

A

B

0
-40
-80
-120
mV

C

D

0.2nA]

0
-40
-80
-120
mV

1min

Figure 7.8. Intracellular recording of an egg fertilised in sodium-free solution, with the membrane potential shifted to various values by injection of constant d.c. current. **A**, recording at the resting potential (-40 mV). **B**, recording at a membrane potential more hyperpolarised than the equilibrium potential for potassium (-100 mV). **C**, recording at a membrane potential near the equilibrium potential for potassium (-80 mV). **D**, recording at a depolarised membrane potential (-20 mV). The responses become depolarised in **B** and disappear in **C**. Upper traces, current; lower traces, membrane potential in **A**, **B**, **C** and **D**.

Membrane Potential Current

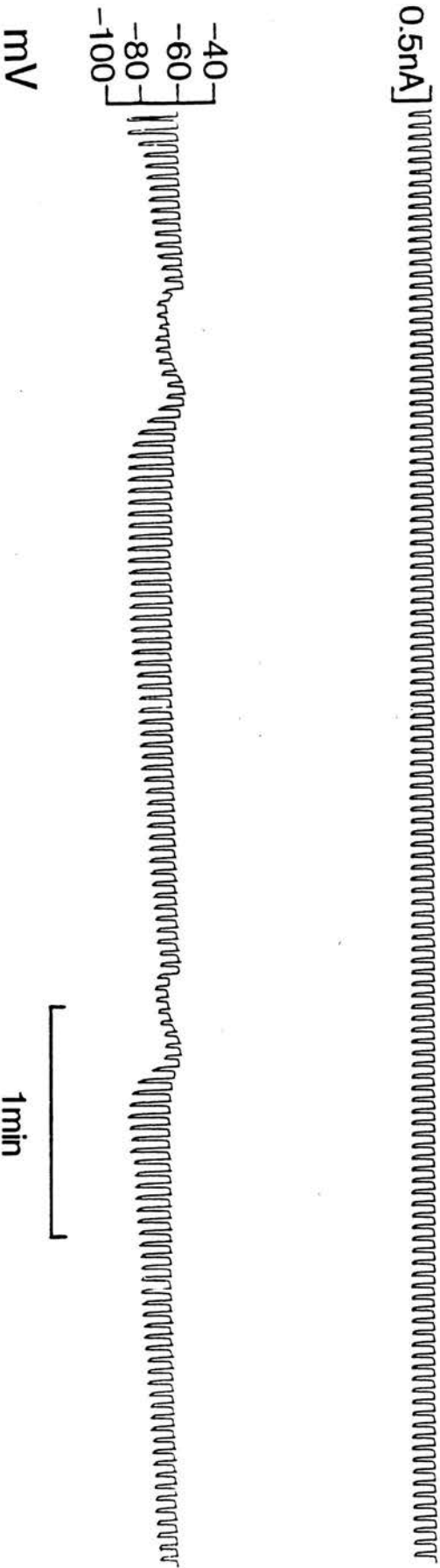


Figure 7.9. Example of a depolarisation and accompanying increase in membrane conductance prior to the hyperpolarising responses induced by sperm. Upper trace, current; lower trace, membrane potential.

The reversal potential for the first two to four hyperpolarisations in sodium-free solution was estimated for 6 eggs. This value, -83.4 ± 8.6 mV ($n=18$) was in good agreement with the reversal potential obtained in normal solution. A slight increase in the duration of hyperpolarising responses was found in Na^+ -free solution (9.7 ± 2.1 s, $n=18$) compared to normal solution (6.9 ± 2.9 s, $n=32$).

One characteristic of fertilisation in sodium-free solution was that a rise in conductance before each hyperpolarising response was always seen, but only at hyperpolarised membrane potentials or after treatment with small concentrations of La^{3+} present in the bath. This is apparent in Fig.7.8 B and C, but a clearer example of this is shown in Fig.7.9. A steady d.c. current was injected into the cell to produce a membrane potential of -60 mV; the steady increase in conductance prior to the hyperpolarisation is accompanied by a small depolarisation (about 5 mV). A decrease in the amplitude of the hyperpolarisations was observed comparing sodium-free (14.5 ± 2.3 mV) to normal solution (31.5 ± 5.3 mV). Although some of this decrease can be accounted for by considering the hyperpolarisation of the membrane in Na^+ -free solution, a genuine inhibition of the conductance increase is apparent.

The combination of Na^+ -free solution and La^{3+} produces a dramatic prolongation of the hyperpolarising response. A comparison of the effects of the same concentration of lanthanum on sperm-induced hyperpolarisations in normal and sodium-free solution was made. Application of lanthanum to an egg in normal solution after insemination produced a prolongation of the sperm-induced hyperpolarisations (see above). Three hyperpolarisations were recorded from an egg before application of La^{3+} (1 mM), with a duration of 7.2 ± 0.7 s ($n=3$). The fourth hyperpolarisation was prolonged by a factor of three compared to the previous three, to 21 s (Fig. 7.10).

This prolongation of the hyperpolarisation produced by La^{3+} was dramatically enhanced by performing this experiment in Na^+ -free solution. In another egg, six hyperpolarisations were recorded before the application of lanthanum, with a duration of 10.4 ± 2.3 s (Fig.7.11 A). The seventh hyperpolarisation occurred in the presence of lanthanum; there was no recovery of membrane potential or input resistance for 10 min, until the egg was washed with normal solution (Fig.7.11 B). After washing, the membrane potential recovered but the input resistance remained low.

Current

0.1nA]

Membrane Potential

-20
-40
-60
-80
mV

1mM La^{3+}

1min

Figure 7.10. The effects of 1 mM lanthanum on the duration of the hyperpolarising responses in normal solution. On addition of 1 mM lanthanum to the solution bathing the egg (indicated by arrow), a three-fold increase in the duration of the response occurs. This was reversed by washing with normal solution for at least 5 min (not shown). Upper trace, current; lower trace, membrane potential.

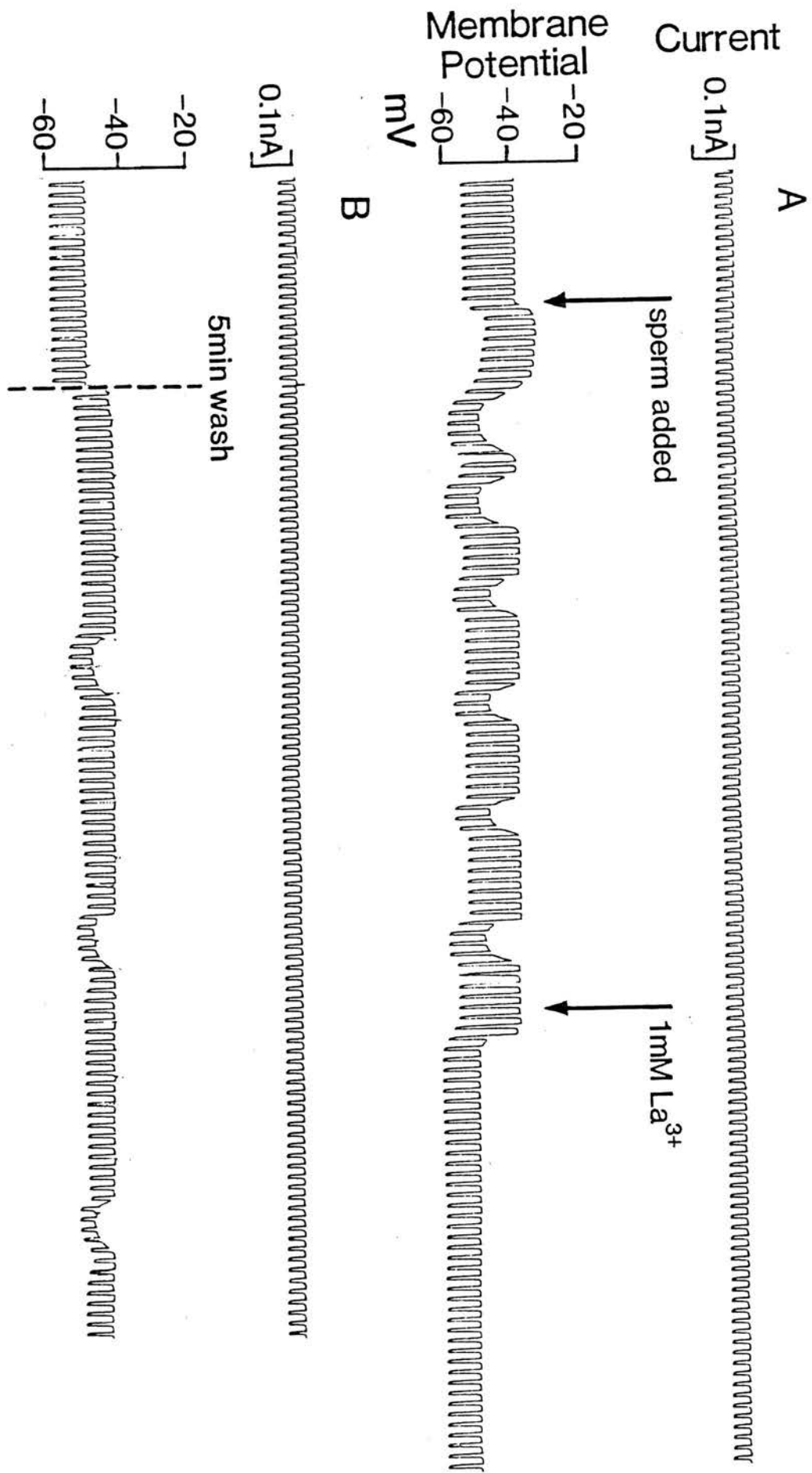


Figure 7.11. The effects of 1 mM lanthanum on the sperm-induced hyperpolarisations of an egg fertilised in sodium-free solution. A, sperm were added at the time indicated by the first arrow and six hyperpolarisations recorded before the addition of lanthanum (second arrow). A prolonged hyperpolarisation occurred until the egg had been washed for 5 min with normal solution (B). Upper traces, current; lower traces, membrane potential in A and B.

This is a common occurrence in fertilised eggs, the input resistance decreases in the course of a long recording, possibly due to activation of a maintained Ca^{2+} -mediated K^{+} conductance (Miyazaki and Igusa, 1982). However, the effects of Ca^{2+} channel blockers mentioned above may suggest a different basis for the hyperpolarisations and the hyperpolarising shift and accompanying ~~the~~ increase in conductance. The hyperpolarising responses resumed shortly after recovery of the membrane potential. They were slightly prolonged compared to responses before lanthanum application; this was probably due to incomplete washout of lanthanum from the chamber, as the frequency of responses was also decreased.

The reversal potential for the prolonged hyperpolarisation in sodium-free plus lanthanum solution was - 97.3 mV, compatible with the mean reversal potential for the previous four hyperpolarisations in this cell, -91.6 ± 3.9 mV.

E The frequency of hyperpolarising responses during fertilisation is dependent on pH_o .

Reducing the pH of the solution bathing unfertilised hamster eggs produces a small depolarisation of the membrane and a decrease in input resistance (Section 3). The effect of reducing the pH on the electrical properties of the hamster egg during fertilisation was examined. The resting membrane potential and input resistance was little affected by low pH, giving values of -23.3 ± 6.2 mV and 391.7 ± 89.5 M Ω , respectively.

In contrast, the frequency of the sperm-induced hyperpolarisations decreased ($n=4$) and was occasionally abolished all together ($n=2$) after perfusing with pH 6.5 solution.

Repetitive hyperpolarisations were initiated by addition of sperm and an indication of their frequency obtained. Fig.7.12 (A and B) illustrates the results from one such experiment; the frequency of the hyperpolarisations was decreased from 5.5 HR/5 min to 1.2 HR/5 min by perfusing with pH 6.5 solution

Little change was observed in the amplitude of the responses in pH 6.5 solution compared to pH 7.5. The reversal potentials for the responses in figure 12 in pH 7.5 were -89.0, -74.7, -77.0 and -80.1 mV, while the reversal potentials for the two responses at pH 6.5 were -77.8 and -80.8 mV. Additionally, low pH_o abolished the hyperpolarising shift. These effects were reversible by washing the egg with normal solution.

By raising the pH of the solution bathing the same egg, the frequency of the sperm-induced hyperpolarisations was increased again to 4 HR/5 min (Fig.7.12 C). A prolongation of the

hyperpolarisations was also observed, from 6.4 ± 1.4 s (n=4) in normal solution, to 10 and 18 s at pH 8.5. Again this effect was reversible by washing the egg with a solution of pH 7.4.

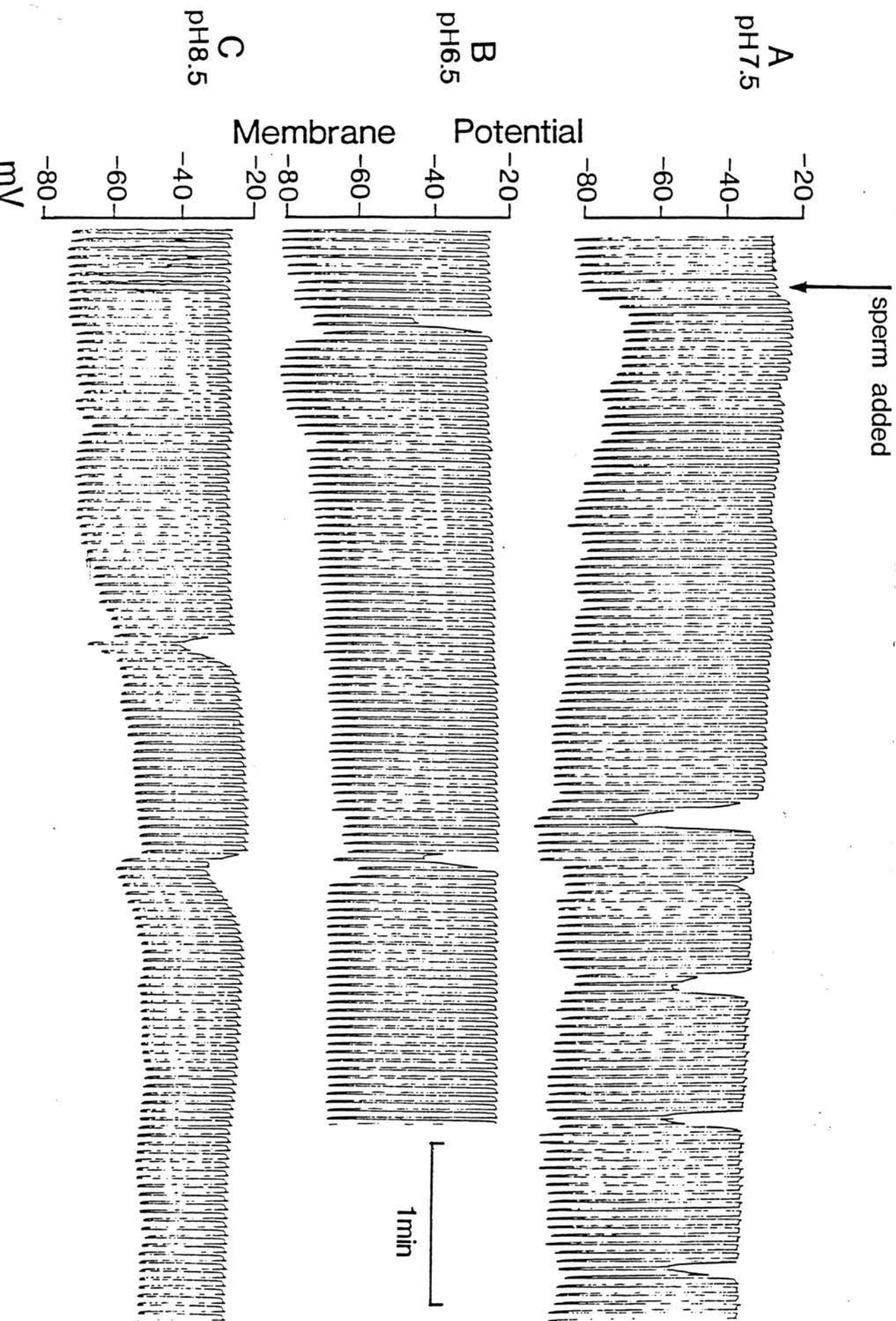


Figure 7.12. Effects of the pH of the solution bathing the egg on the frequency and duration of hyperpolarising responses. A, sperm added at time indicated by arrow and responses recorded at normal pH. B, frequency of responses was decreased when the pH was lowered to 6.5. A depolarisation of the membrane also occurred. C, this depolarisation was reversed by perfusing the egg with pH 8.5. The responses in pH 8.5 were more prolonged than in normal solution, and the frequency was increased above that recorded at pH 6.5. Upper traces, current; lower traces, membrane potential in A, B and C.

F Abolition of the hyperpolarising responses by DNP.

The addition of 2,4, dinitrophenol (DNP) to the solution bathing an inseminated egg resulted in a steady depolarisation of about 5 mV and a 30 % decrease in input resistance; these changes in the membrane potential and input resistance were not as dramatic as the changes in unfertilised hamster eggs induced by DNP (Section 3).

The presence of DNP abolished the sperm-induced hyperpolarisations (n=3). Recovery of the membrane potential and resistance values occurred after prolonged washing of the egg with normal solution. However, the hyperpolarisations in normal solution, after treatment with DNP, were smaller and less frequent than those before DNP treatment.

Discussion.

The fertilisation potential of the hamster egg.

In some species, the egg fertilisation potential has an early depolarising phase, due to an increase in permeabilities to Na^+ and Ca^{2+} . This depolarising phase lasts for several minutes in echinoderm (Miyazaki and Igusa, 1981) echiuran (Jaffe, Gould-Somero, and Holland, 1979) and tunicate (Kozuka and Takahashi, 1981) eggs. This was thought to act as a fast electrical block to polyspermy.

Recently, however, Hinkley (1986) demonstrated that even in sea urchin eggs clamped at negative potentials, only one sperm fuses with the egg. Thus a depolarising fertilisation potential may not necessarily indicate an electrical block to polyspermy. No such large depolarising phase was recorded in the hamster egg.

In conjunction with the severe polyspermy observed in zona-free eggs (independent of the potential they are held at), this is further evidence that an electrical block to polyspermy does not exist in the hamster egg. The major block to polyspermy in the hamster probably resides in the zona pellucida; after cortical granule exocytosis induces the 'zona reaction', the zona becomes 'refractory' to sperm penetration (Wolf, 1981).

The fertilisation potential of the hamster egg is a series of transient hyperpolarisations of the egg plasma membrane. The reversal potential for these responses was compatible with the equilibrium potential for K^+ . Miyazaki and Igusa (1982) showed that this reversal potential was unaltered by the removal of external chloride, and that the potassium channels underlying the hyperpolarisations were activated by a rise in the intracellular free Ca^{2+} concentration. They proposed that a release of Ca^{2+} from intracellular Ca^{2+} stores was responsible for this rise in

$[Ca^{2+}]_i$, and that these stores were re-stocked by influx of Ca^{2+} across the plasma membrane. Such a release would then activate the Ca^{2+} -activated K^+ channels, hyperpolarising the membrane. However, no depolarisations (due to a possible Ca^{2+} influx) were usually seen just prior to the hyperpolarisations in normal solution, in this study. This was the case even when the membrane was current clamped to hyperpolarised potentials (-100 mV), where a greater electrochemical gradient for Ca^{2+} would exist. However, in the Na^+ -free experiments, a depolarisation accompanied by a rise in conductance was occasionally observed, especially at hyperpolarised potentials.

The input conductance of the membrane of this egg slowly rose from a minimum value recorded a few seconds after the hyperpolarisation (2 nS) to a maximum just prior to the hyperpolarisation (55 nS). If this rise in conductance due to a Ca^{2+} influx, it is only noticeable when the background conductance due to Na^+ influx has been removed. It is likely that the Ca^{2+} for the rise in $[Ca^{2+}]_i$ associated with each hyperpolarisation comes from intracellular stores, which are in turn dependent on $[Ca^{2+}]_o$ for re-stocking.

The dependence of the fertilisation potential on $[Ca^{2+}]_o$ was demonstrated by replacing Ca^{2+} with Mg^{2+} and Ba^{2+} . Neither of these ions could sustain the hyperpolarisations. The inability of Ba^{2+} to sustain the response is not due to a lower permeability for Ba^{2+} compared to Ca^{2+} , as described for voltage-gated Ca^{2+} channels of mouse and sea urchin eggs (Okamoto, Takahashi and Yamashita, 1977): injection of Ba^{2+} (or magnesium) into hamster eggs failed to activate a potassium conductance (Georgiou et al, 1987). In contrast, Sr^{2+} injection produced a hyperpolarisation of the hamster egg membrane. Similarly, Igusa and Miyazaki (1983) demonstrated that Sr^{2+} could sustain repetitive hyperpolarisations in fertilised hamster eggs. The influx of Ca^{2+} into the egg probably does not involve the voltage-gated Ca^{2+} channels previously reported in hamster eggs, as these are inactivated at the recorded resting membrane potentials.

To investigate the Ca^{2+} channels involved in influx at the resting potential, different Ca^{2+} channel blockers were applied. The relative potency of several blockers can be used to distinguish types of Ca^{2+} channels. In order of potency in blocking the hyperpolarisations (and presumably Ca^{2+} influx) lanthanum comes first, followed by cadmium and then cobalt. This particular order of potency for blockers resembles the Type I rather than the Type II channel profile for mouse neuroblastoma cells (Narahashi, Tsunoo and Yoshii, 1987). There are several important differences, however, as the neuroblastoma channels are voltage-sensitive. The possibility exists that some of these Ca^{2+} channel blockers have a direct inhibitory on Ca^{2+} -activated potassium channels. Such possibilities could be investigated by the use of single channel analysis to characterise the Ca^{2+} channels.

Various inhibitors of Ca^{2+} extrusion prolong the duration of hyperpolarising responses.

As well as its action as a Ca^{2+} channel blocker, decreasing the frequency of the hyperpolarising responses, La^{3+} produced a prolongation of the hyperpolarising responses. The action of La^{3+} was dose-dependent; the effects on Ca^{2+} influx were apparent at a lower concentration than the effects on the duration of hyperpolarising responses. The reversal potentials of the sperm-evoked responses were unchanged by lanthanum, suggesting that the potassium conductance was unaffected by this treatment. The La^{3+} effect was also reversible after washing with normal solution for a sufficient period.

The reversible effect of La^{3+} on the duration of the hyperpolarising responses can be attributed to its action as an inhibitor of Ca^{2+} extrusion; lanthanum has been shown to inhibit Ca^{2+} extrusion in hamster eggs (Georgiou et al, 1987a). The temporary inhibition of these hyperpolarisations by La^{3+} did not interfere with the process of fertilisation, as determined by the appearance of decondensing sperm heads or male pronuclei ($n=4$).

The effect of La^{3+} in decreasing the frequency of the hyperpolarising responses is rather surprising; although it blocks the voltage-gated Ca^{2+} channels, the results from Section 5 would suggest that it has no effect on non-voltage gated Ca^{2+} influx (La^{3+} cannot prevent the pH_o -induced hyperpolarisation). It may be that any inhibitory effect of La^{3+} on non-voltage gated Ca^{2+} influx is overcome by high pH , and the electrical properties of the fertilised egg are sufficiently different to account for this discrepancy.

In an attempt to inhibit the Na^+ - Ca^{2+} exchange of the hamster egg, fertilisation was performed in a Na^+ -free solution. Choline was used to replace Na^+ . A decrease in the amplitude of the hyperpolarising responses was obtained with this replacement, even taking into account the hyperpolarised membrane potential in Na^+ -free solution, probably due to a block of Ca^{2+} -mediated K^+ channels. Removal of external sodium produced only a slight increase in the duration of the hyperpolarisations compared to normal solution. As the method of measuring duration of response depends on the amplitude of the hyperpolarisation (Section 2), it is likely that a larger prolongation of the hyperpolarisations would be apparent if the membrane was not so hyperpolarised. On the other hand, it is possible that inhibition of the Na^+ - Ca^{2+} exchange by Na^+ removal did not dramatically reduce the ability of the egg to extrude Ca^{2+} as the Ca^{2+} pump was still functional. This would be in accordance with the results of Sections 5, on the effects of high pH_o .

To examine this, lanthanum was applied in the presence and absence of sodium. The results of Fig.7.10 suggest that the combination of inhibiting both extrusion systems is potent in

producing a rise in intracellular Ca^{2+} , activating the Ca^{2+} -mediated potassium channels until perfusion with normal solution.

In Na^+ -free solution, with no lanthanum present, the Ca^{2+} pump may therefore increase its work-load, extruding Ca^{2+} when the Na^+ - Ca^{2+} exchange is blocked. This would result in a smaller prolongation than if there was no Ca^{2+} pump present and a Na^+ - Ca^{2+} exchange was the only mechanism for Ca^{2+} extrusion.

Alternatively, the greater effect of lanthanum than Na^+ -free solution in prolonging the hyperpolarisations may just reflect a greater involvement of the Ca^{2+} pump than the Na^+ - Ca^{2+} exchange in the recovery phase of the sperm-induced hyperpolarisations, rather than a compensatory action of the Ca^{2+} pump. The latter explanation would be supported by the results in Section 4 on Ca^{2+} injection, where lanthanum again has a greater effect on the recovery phase of transient hyperpolarisations.

Although qualitatively similar, the effect (inhibition of Ca^{2+} extrusion) of Na^+ -free solution plus lanthanum in fertilised eggs is much more potent than the combination of Na^+ -free and pH_o solution in unfertilised eggs (Section 4). In unfertilised eggs, these treatments only prolong the hyperpolarisations due to Ca^{2+} injection. In fertilised eggs, in the presence of Na^+ -free solution and lanthanum, a steady hyperpolarisation was obtained (Fig. 7.11). This result is compatible with the observations by Igusa and Miyazaki (1983) on the differences between Ca^{2+} injection in fertilised and unfertilised eggs. They report that a smaller injection current is required to produce a large all-or-none response in fertilised eggs compared to unfertilised eggs.

It is likely that in fertilised eggs an amplification system is activated, possibly similar to the one discussed in Section 6. Raising the pH_o of the solution bathing the egg prolongs the duration of the sperm-induced hyperpolarisations in a similar way to the application of lanthanum; high pH_o has been shown to inhibit Ca^{2+} extrusion in the hamster egg (Georgiou et al, 1988).

In contrast to lanthanum, however, a raised pH_o increased the frequency of the hyperpolarising responses. This may be due to a direct effect of raised pH_o on non-voltage gated Ca^{2+} channels, causing an enhanced influx of Ca^{2+} into the egg; high pH increases the influx of Ca^{2+} through voltage-gated Ca^{2+} channels in these eggs (Georgiou et al, 1988) but these channels are inactivated at the resting potential.

Lowering pH_o had little effect on the time-course of the hyperpolarisations, but decreased their frequency of occurrence. This suggests an inhibitory effect of pH on the influx of Ca^{2+} rather than on the extrusion systems. There may be a relation between the low pH_o effect and the DNP effect, if the latter is producing its effect as a proton ionophore. However, the DNP effect should be

interpreted cautiously, as one result of proton ionophore activity is the inhibition of oxidative phosphorylation. During fertilisation, the sperm somehow initiates an increase in the metabolic activity of eggs; unfertilised mouse eggs are metabolically depressed compared to fertilised mouse eggs (Leese, Biggers, Mroz and Lechene, 1984). Inhibition of this rise in metabolic rate by DNP could retard the fertilisation processes in general (for example, protein synthesis). It may be that sperm-evoked hyperpolarisations in the hamster egg are particularly sensitive to metabolic inhibition. Evidence exists for involvement of a G-protein in the process leading to activation of Ca^{2+} -mediated K^+ channels; injection of GTPyS into hamster eggs evokes repetitive hyperpolarisations (Miyazaki, 1987b). If the energy required for this G-protein to function is inhibited by DNP, and this protein is involved in evoking repetitive hyperpolarisations, then the responses would be inhibited

SECTION 8 Preliminary single channel recordings from the hamster egg.

Single channel recordings from invertebrate and amphibian eggs have been made for some time (Fukushima, 1981; Jaffe, Kado and Muncy, 1985). In contrast, there are few reports of use of the patch-clamp recording technique to investigate mammalian eggs. The whole-cell recording mode has been used to investigate macroscopic Ca^{2+} currents in the mouse egg (Peres, 1987). The first application of single channel recording techniques to mammalian eggs was by Bountra and Martin (1987), on zona-free mouse eggs. They reported a high conductance channel (130 pS) permeable to K^+ under cell-attached mode, but comment that further work is required to identify the channel.

This Section is the first report of single-channel recordings from a hamster egg. The excised inside-out patch conformation (Hamill et al., 1981) was used. With this technique, the composition of the solution bathing the inner surface of the membrane could be controlled.

These preliminary recordings may reveal the existence in the hamster egg of a novel Ca^{2+} -activated K^+ channel with a low single channel conductance, acting as an outward rectifier. Additionally, the results suggest that intracellular ADP may play a role in regulating the activity of this channel in the hamster egg.

Results.

The rate of success in obtaining channel activity after giga-seal formation (between 1 and 3 G Ω) was rather low in these preliminary experiments, even after excision of the membrane patch. This could have been due to a low channel density. The situation was improved when the solution bathing the intracellular membrane surface of excised patches included Mg^{2+} ; in Mg^{2+} -free solution, a 15 % success rate was seen (3/20 giga-seals), while with 1.0 mM Mg^{2+} in the intracellular solution, the success rate was 50 % (11/22 giga-seals). The amount of MgCl_2 in the extracellular solution (intrapipette solution) was reduced in compensation for the intracellular solution because Mg^{2+} is chelated to a certain extent by EGTA (Findlay, 1987). Accordingly, the intrapipette solution and the extracellular solution also contained 1.0 mM Mg^{2+} .

It was observed that once a giga-seal had been obtained in the cell-attached conformation, the patch need not be active until the membrane was excised. In some cells, the channel activity did not appear until 20 min after the membrane was excised. For this reason, experiments were performed on excised inside-out patches of membrane. Additionally, this allowed control of the concentration of various ions such as Ca^{2+} , K^+ and Na^+ bathing the inner membrane surface. Although insufficient data has been collected to define the channels observed as Ca^{2+} -activated K^+

channels, there is some circumstantial evidence to suggest this possibility. The currents reversed at 0 mV with 140 mM KCl on both sides of the patch, and no Na⁺ was present, so the ionic basis for the current is either K⁺ or Cl⁻. The activity of the patches was dependent on [Ca²⁺]_i; there is evidence of a Ca²⁺-activated K⁺ channel in the hamster egg (Miyazaki and Igusa, 1982) while there is no data for a Ca²⁺-activated Cl⁻ channel in the hamster egg.

Properties of the channel recorded in the hamster egg.

A Dependency of Ca²⁺-activated K⁺ channel on membrane potential.

The conductance of the Ca²⁺-activated K⁺ channel is decreased at hyperpolarised membrane potentials compared to depolarised membrane potentials. The decrease in the current amplitude was obvious when the records of a single Ca²⁺-activated K⁺ channel were compared at positive and negative membrane patch potentials (Fig.8.1 A).

The single channel current was plotted against membrane potential for the channel described in Fig.8.1 A to produce the I-V relation plotted in Fig.8.1 B. This I-V curve shows an outward rectification. The single channel conductance for this particular channel was 51.9 pS for depolarised values of the membrane patch potential, and 10.4 pS for hyperpolarised membrane patch potentials. The mean conductances for depolarised potentials from four channels was 65.3 ± 12.9 pS. The corresponding value for hyperpolarised membrane potentials was 23.4 ± 11.8 pS. The open probability (P_o) of these channels was dependent on the membrane potential. The same channel has been used to illustrate this dependence on membrane potential (Fig.8.2) at a [Ca²⁺]_i of 4 μM. The values of channel open probability were obtained using the data processing package described in Section 2. The channel open probability increased with depolarisation, being lower than 20 % at more hyperpolarised membrane potentials than the zero current level. The maximum channel open probability was about 60 % for a membrane patch potential of + 50 mV, with a [Ca²⁺]_i of 4 μM. However, some channels had larger values of P_o than this, at around 90 % at + 60 mV.

B Dependency of channel activity on [Ca²⁺]_i.

No channel activity was observed in the channels reported here when the free [Ca²⁺] at the intracellular side of the membrane patch was 0.1 μM or lower. Channel activity was always observed at 0.5 μM, suggesting that the threshold of activation for these channels lies between the two values. The recordings in these experiments were obtained with [Ca²⁺]_i of 0.5 and 4 μM.

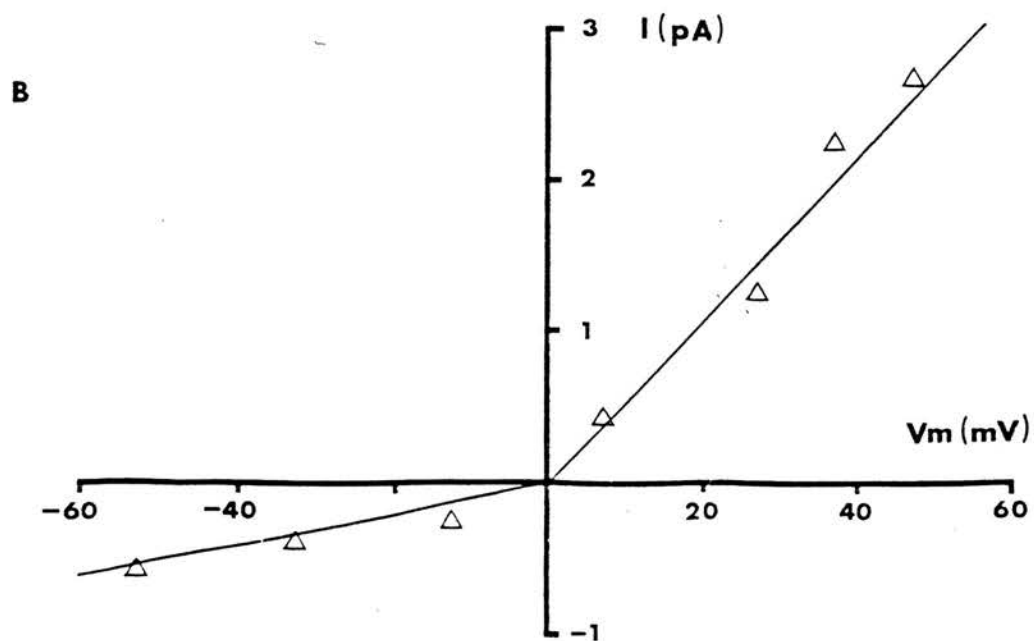
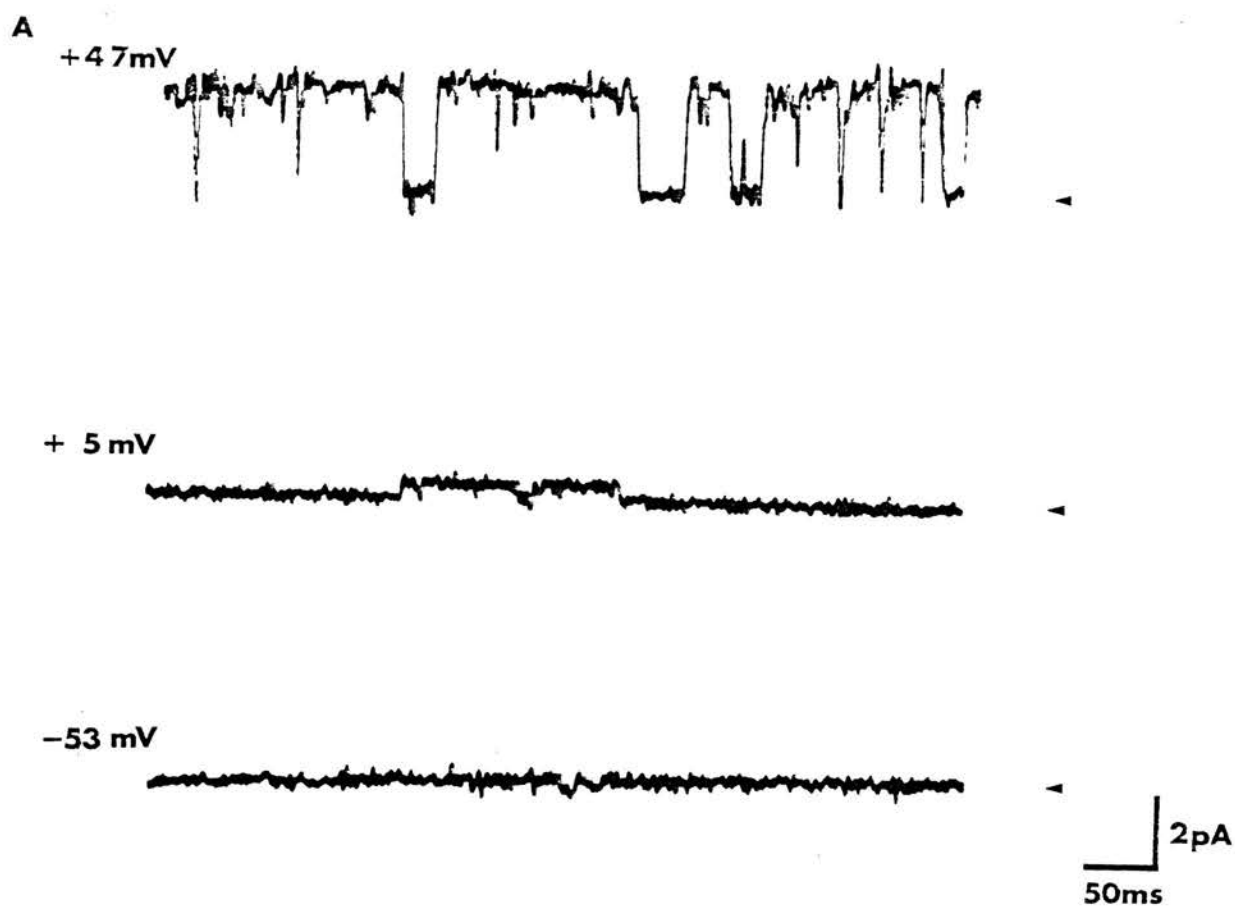


Figure 8.1. A patch clamp recording of a single channel, in the inside-out patch conformation, obtained from a hamster egg membrane. **A**, the patch was held at various membrane potentials; numbers to the left of each trace refer to the holding potentials. The membrane was exposed to symmetrical KCl and MgCl_2 . The internal concentration of Ca^{2+} was $4\ \mu\text{M}$, the intrapipette solution contained $5\ \text{mM}\ \text{Ca}^{2+}$. The arrows indicate the zero current level (closed channel state). Currents were filtered at 500Hz . **B**, the single channel I-V relationship obtained from the same patch as the recording in **A**, displaying outward rectification.

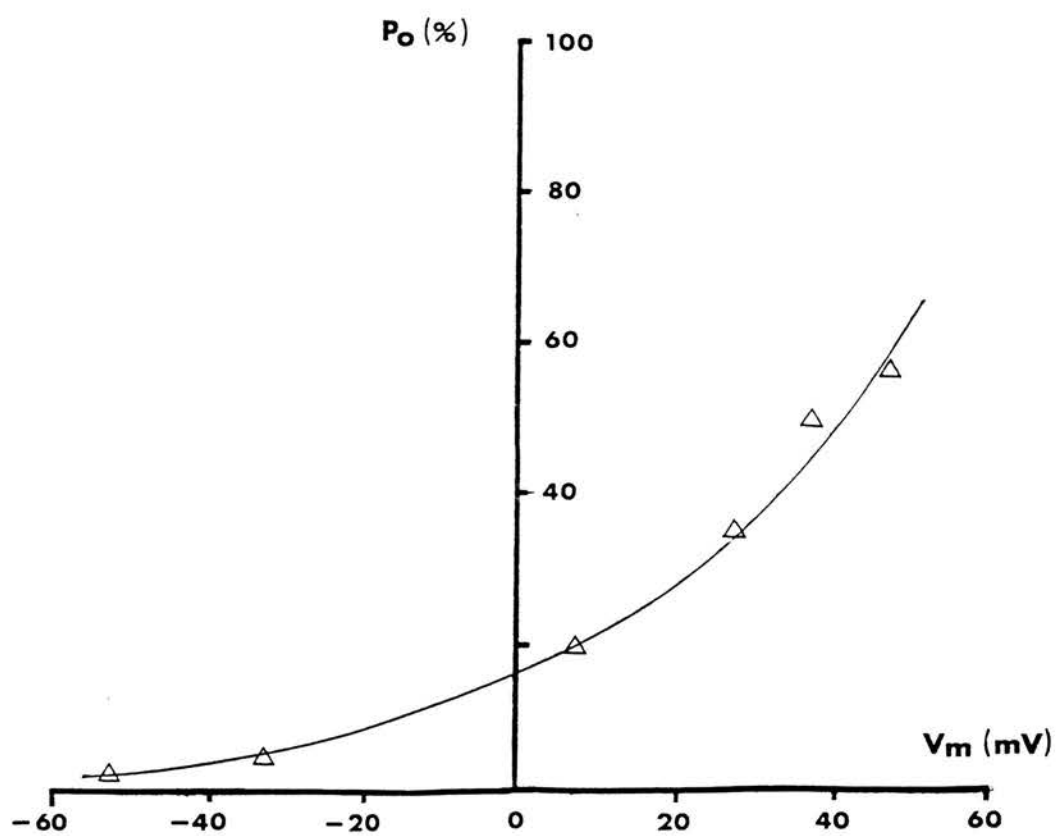


Figure 8.2. Potential dependence of the open probability (P_o) of the channel illustrated in Fig.8.1.
The P_o increases with depolarisation of the patch.

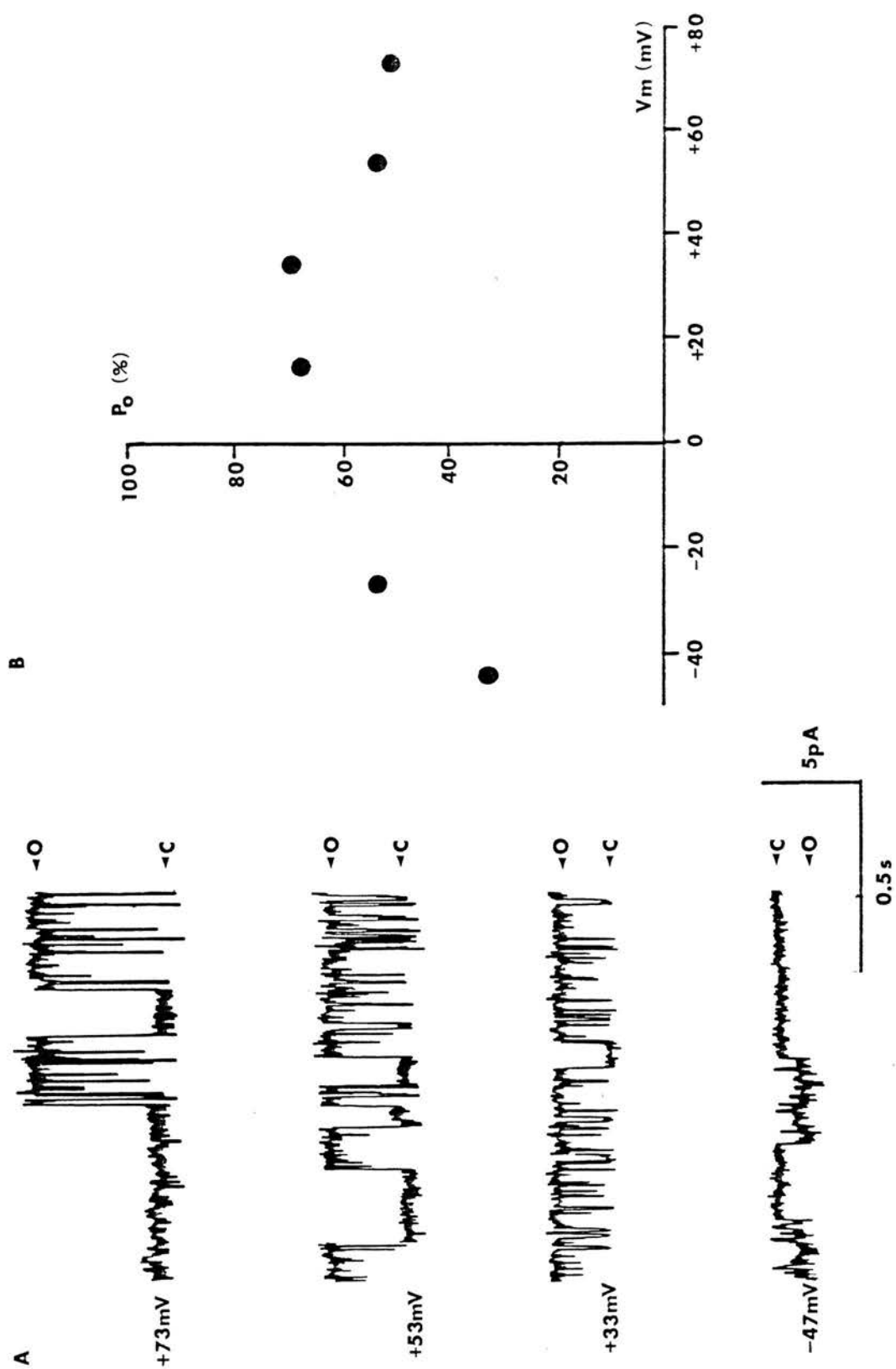


Figure 8.3. A decrease in P_o on depolarisation to positive membrane potentials occurs in some patches. **A**, The channel activity was higher at + 33 mV than at more hyperpolarised or depolarised membrane potentials, with 4 μM Ca^{2+} bathing the intracellular face of the patch. The composition of solutions was identical to that for the channel described in Fig.8.1. **O** and **C** represent open and closed channel states. Numbers to the left of each trace refer to the holding potential. Currents were filtered at 500Hz. **B**, the relation between the membrane potential and P_o for the channel described in **A**.

The dependence of channel activity on $[Ca^{2+}]_i$ varied from cell to cell. In some membrane patches, a decrease in the channel open probability (P_o) at a depolarised membrane potentials was observed with a high $[Ca^{2+}]_i$. Illustrated in Fig.8.3A is a recording of channel activity in a patch with 4.0 μM Ca^{2+} in the solution bathing the intracellular face. In addition to the obvious outward rectification, the time spent by the channel in the open state is dependent on the membrane potential. The channel was open more frequently at +33 mV than at either more hyperpolarised (-47 mV) or depolarised (+53 and +73 mV) levels. The P_o of this channel was plotted against membrane potential for six holding potentials (Fig.8.3B), and was found to increase from a minimum of 36.1 % at -47 mV to a maximum of 70.1 % at +33 mV. It then decreased again, to 52.8 % at +73 mV.

C Multiple channel openings.

The low success rate in obtaining channels in the hamster egg might be considered paradoxical in the light of multiple unitary channel openings recorded in a single patch. An excised membrane patch could contain as many as 4 channels of unitary conductance; it may be that the channels occur in clusters. Not all of these channels were usually open at one time, but after 1 to 2 s, all channels had usually opened with 4 μM Ca^{2+} bathing the intracellular surface of the patch. A recording from one of three such patches is illustrated in Fig.8.4A.

Photographs from the storage oscilloscope screen were taken of recordings from a patch containing 4 channels after (i) a single trace of the current and (ii) showing multiple superimposed traces of these channels. This patch was held at a membrane potential of +40 mV, where each channel had a unitary current amplitude of 2.4 pA. This is illustrated in Fig. 8.4B, a current amplitude histogram of these four channels, produced by the soft-ware package described in Section 2.

D Multiple conductance states.

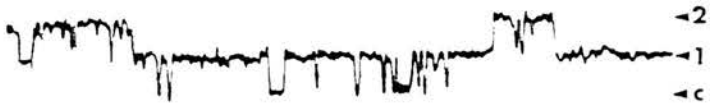
In recordings from some patches, at least one conductance state, distinct from the channel main state, was observed. Here, the channel main state refers to the state in which the channel is open for most of the time, while the subconductance state refers to an open state of smaller conductance and lower incidence.

Examples from one such patch are shown in Fig. 8.5. The arrows indicate the position of the second state. There are several reasons why this second open state is unlikely to be due the presence of two similar channels. Firstly, this second state is not half the amplitude of the main state, as would be expected if two similar channels were in the same patch. For example, in

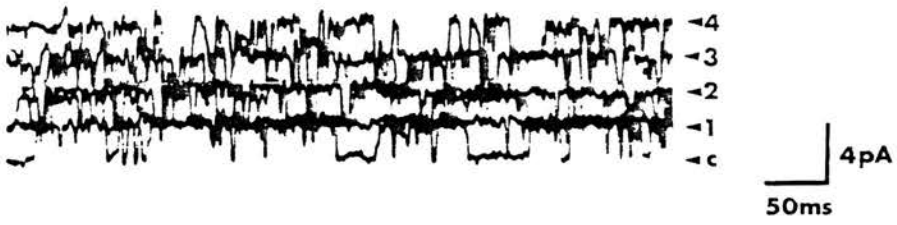
Fig.8.4, where four channels were obtained in one patch, the single channel current amplitude was the same for each channel.

A +40mV

i



ii



B

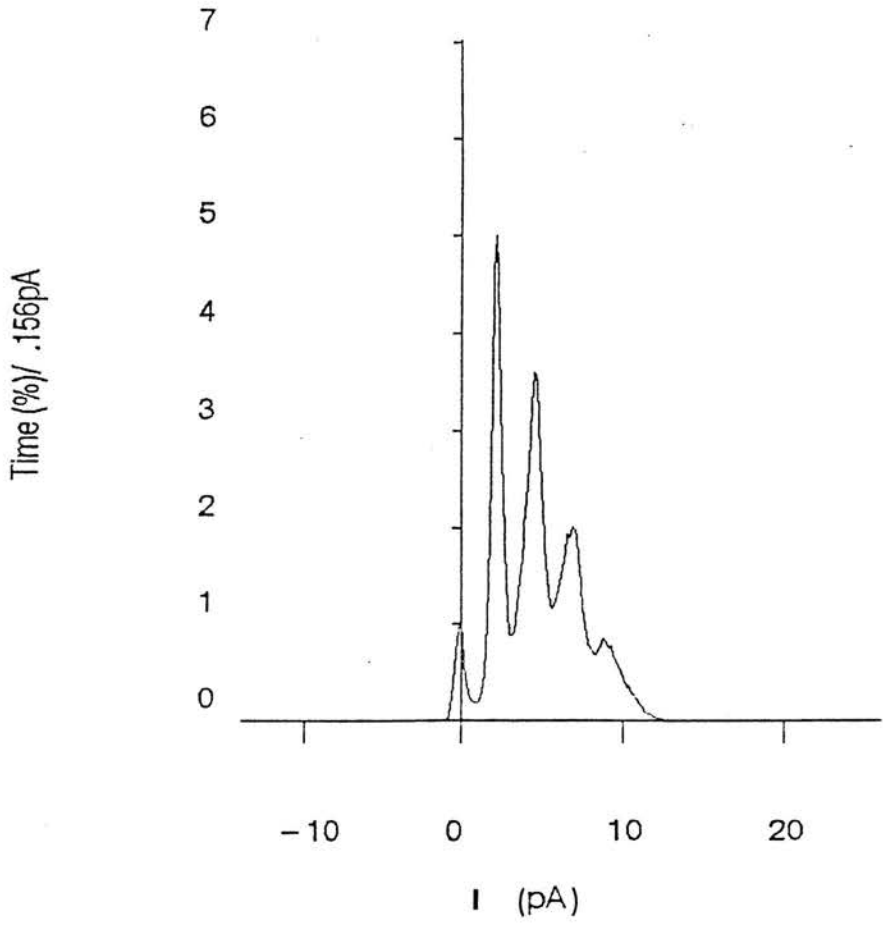
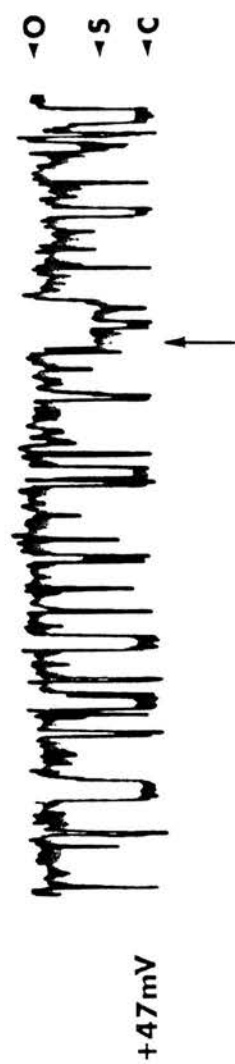


Figure 8.4. An example of multiple channels occurring in one patch. **A.** channel recordings from four channels in a single inside-out patch of hamster egg membrane. The patch was bathed with symmetrical KCl and MgCl_2 , with $4\mu\text{M Ca}^{2+}$ bathing the intracellular face of the patch. (i) shows a single trace of channel current. (ii) shows multiple superimposed traces from the oscilloscope screen. **B.** A current amplitude histogram of the four channels present in the patch described in A was made. The ordinate axis is the number of observations (the percentage of the analysis time that the channels spend in a series of bins with a 0.156 pA binwidth), the abscissa is the amplitude of the single channel current.

A



B

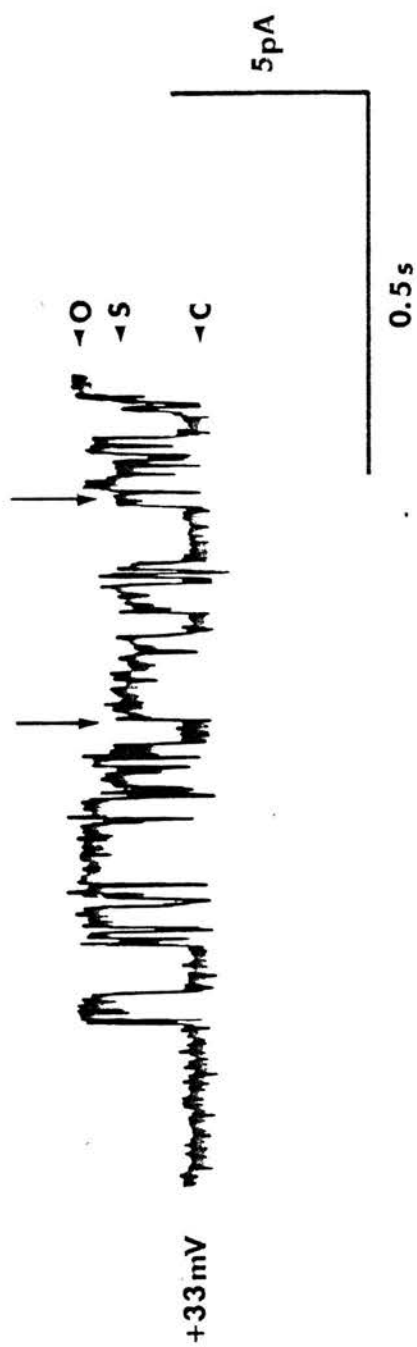


Figure 8.5. Single channel recordings from a channel displaying subconductance states, at two holding potentials, +47 mV (A) and +33 mV (B). The patch was bathed with symmetrical KCl and MgCl₂, with 4μM Ca²⁺ bathing the intracellular face of the patch. Arrows indicate the positions of the substates. At the right of each trace the closed channel state (c), open channel state (o) and the subconductance state (s) are indicated. The substates appear to have different voltage sensitivities than the main state. Currents were filtered at 500Hz.

One way of differentiating the main state and the substate was by using different holding potentials; the size of the current amplitudes for each state could be selectively altered. Hyperpolarising the patch from + 47 mV (Fig.8.4 A) to + 33 mV (Fig.8.4 B) increased the single channel current amplitude for the substate with little effect on the channel main state, suggesting a greater voltage-sensitivity.

Secondly, the frequency of occurrence of the open channel states with smaller current amplitudes was much lower than that for the main channel states.

Thirdly, at different potentials, the probability of observing the proposed substates could be altered. For example, two channels were recorded from a patch held at + 40mV (Fig.8.6A), with two possible substates. A current amplitude histogram was plotted which contained four peaks, corresponding to two main channel open states and two channel substates.

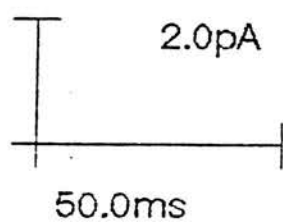
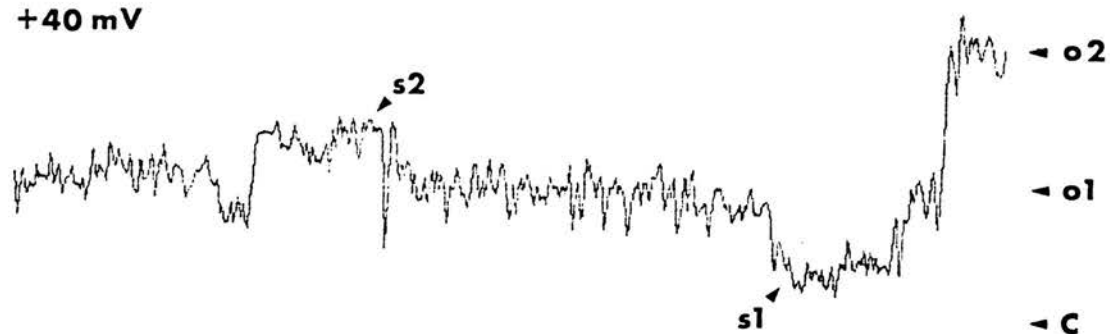
The main state of each channel had a current amplitude of 2.1 pA, while the less-frequent substate had an amplitude of 1.2 pA (Fig.8.6 B). By depolarising the patch to + 60 mV, the single channel current amplitude of each channel increased, and the substates disappeared (Fig.8.7 A). Only two peaks were observed on a current amplitude histogram (Fig.8.7 B), corresponding to the two main open states of each channel, with single channel current amplitudes of 3.7 pA.

E Channel kinetics

The presence of bursts of channel opening and closure seen earlier in Figs.8.1, 8.3 and 8.5 are a characteristic of Ca^{2+} -activated K^{+} channels (Barrett, et al, 1982; Smart, 1987). These bursts are more easily seen at hyperpolarised than depolarised potentials; the P_o is so large at depolarised levels that the channel is open for most of the time easily. Bursts are defined in terms of a gap between bursts of channel opening that is calculated from distributions of shut intervals; all intervals greater than the calculated gap are taken as gaps between bursts.

An example of bursts of opening is shown in Fig.8.8A, in a patch held at + 73 mV. At this potential the single channel current is large; this improves the signal-to-noise ratio, reducing the possibility of noise contributing to the kinetics. The records run consecutively and channel opening is indicated by an outward current (upward). In a burst of channel opening such as this, the channel is in an open state for much of the time.

A +40 mV



B

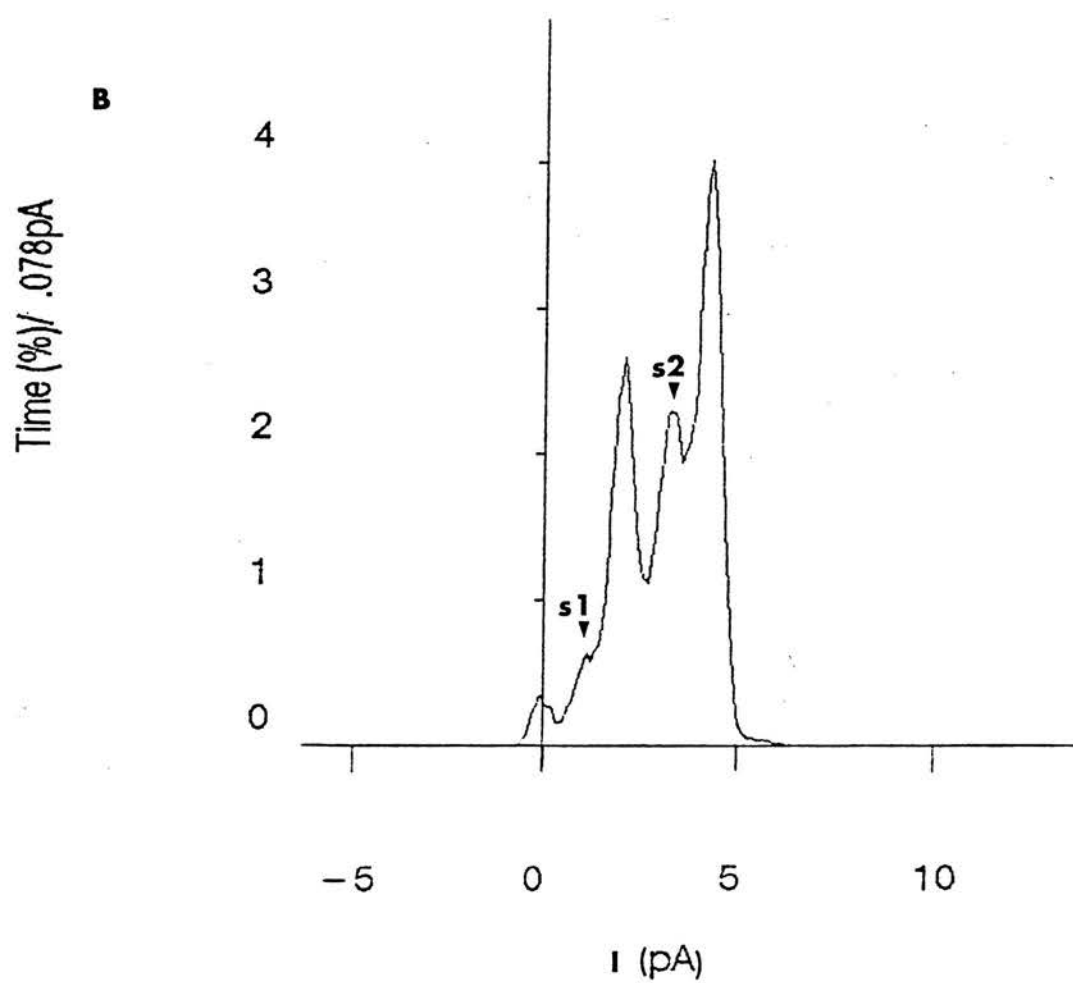


Figure 8.6. A recording from a patch containing two channels with at least one subconductance state each. The patch was bathed with symmetrical KCl and MgCl₂, with 4μM Ca²⁺ bathing the intracellular face of the patch. **A.** The patch was held at +40 mV and channel openings recorded. The main open state of each channel (o1 and o2), the closed state (c) and the substates are indicated on the right of the trace. **B.** A current amplitude histogram of the channels described in A was made, showing two major peaks for the channel main states, and two minor peaks (indicated by the arrows) corresponding to the subconductance states. The ordinate axis is the percentage of the analysis time that the channels spend in a series of bins with a 0.078 pA binwidth, the abscissa is the amplitude of the single channel current. Currents were filtered at 500Hz.

A +60 mV

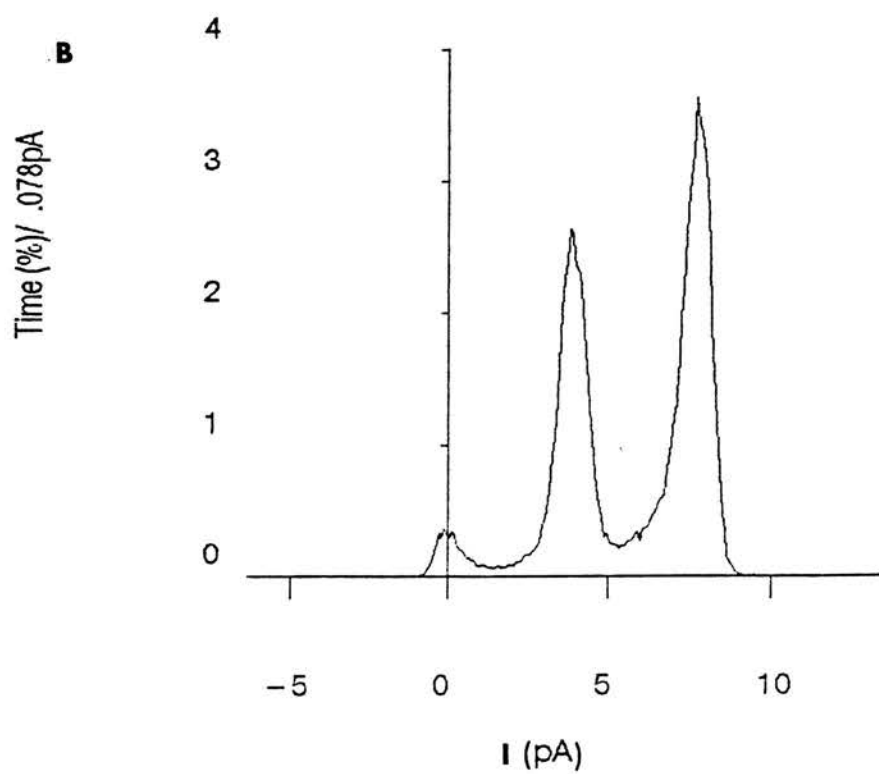
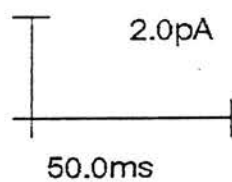
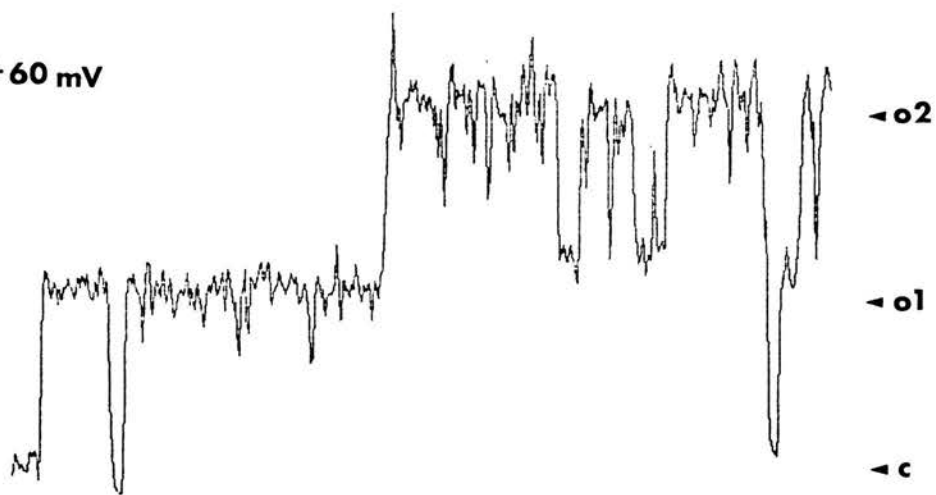


Figure 8.7. Single channel recordings from the same patch described in Fig.8.6, held at a more depolarised potential (+ 60 mV). The patch was bathed with symmetrical KCl and MgCl₂, with 4μM Ca²⁺ bathing the intracellular face of the patch. **A.** The channel does not show any subconductance states, just one open (o1), two open (o2) or both closed (c). **B.** A current amplitude histogram of the channel described in A, held at + 60 mV. The two small peaks seen in the previous current amplitude histogram (Fig.8.6B) of this channel disappear at this more depolarised potential, leaving just the two major peaks. The ordinate axis is the percentage of the analysis time that the channels spend in a series of bins with a 0.078 pA binwidth, the abscissa is the amplitude of the single channel current. Currents were filtered at 500 Hz.

A +73 mV

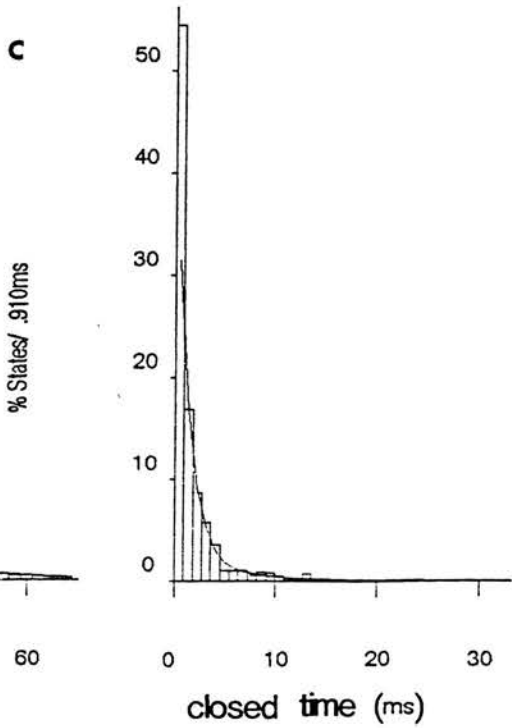
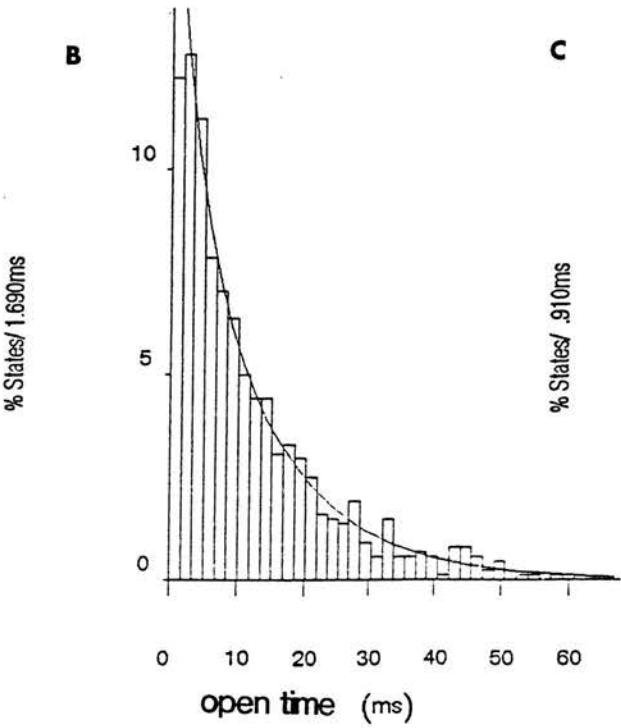
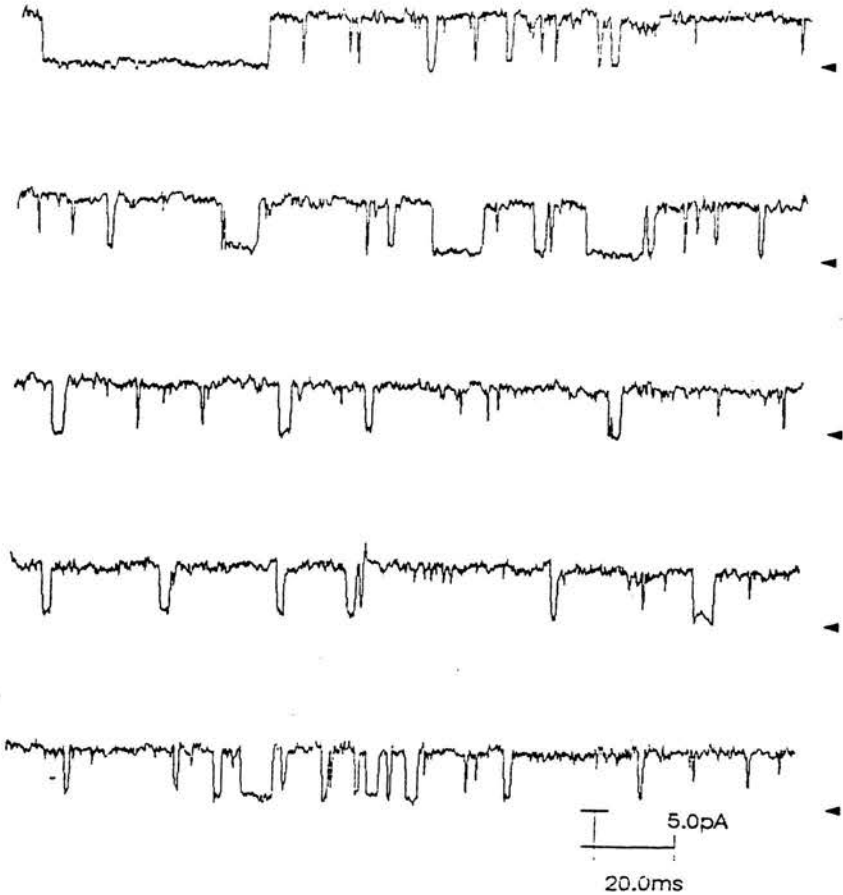


Figure 8.8. Analysis of channel kinetics of a single channel in an excised, inside-out patch of hamster egg membrane. **A.** Bursts of channel opening in a patch held at a membrane potential of +73 mV. Channel opening is upward producing a positive (outward) current. The closed state is indicated by an arrow. The currents were filtered at 1.5 kHz. **B.** An open time histogram of the channel openings described in A, showing the number of channel openings in the time of the analysis. The ordinate is the percentage of channel openings of the channel in bins of 1.69 ms binwidth, the abscissa is limited to a length of 60 ms. Two exponentials were used to describe the distribution of open times. Open times longer than a minute were analysed by hand. **C.** A closed time histogram of the channel held at +73 mV. Two exponentials were used to describe the distribution of the closed times. The patch was bathed with symmetrical KCl and MgCl₂, with 4μM Ca²⁺ bathing the intracellular face of the patch.

The channel open times from this channel were analysed using the soft-ware described in Section 2 to obtain the histogram in Fig.8.8B. This is a plot of the number of channel openings against the time the channel is in the open state. Two exponentials were required to describe the distribution of observed open channel times. These had time constants of 3.14 and 13.0 ms.

Similarly, the channel closed-times were analysed. Two exponentials were used to describe the distribution of channel closed times (Fig.8.8C). The time constants of these exponentials were 1.38 and 6.84 ms, with the mean closed time of the channel of 9.55 ms. When long closures in the order of seconds were observed, they were measured by hand. These closures were occasionally up to 17 min long.

F Modulation of Ca^{2+} -activated K^{+} channel activity by ADP.

i. The channel open probability was enhanced by ADP.

In channels recorded from patches with 0.1 mM ADP bathing the intracellular face of the membrane ($n=4$), the dependence of P_o on membrane potential was reduced compared to that in the absence of ADP. For example, at hyperpolarised membrane potentials, the decrease in P_o seen in the absence of ADP, was not apparent in the presence of ADP (Fig.8.9A).

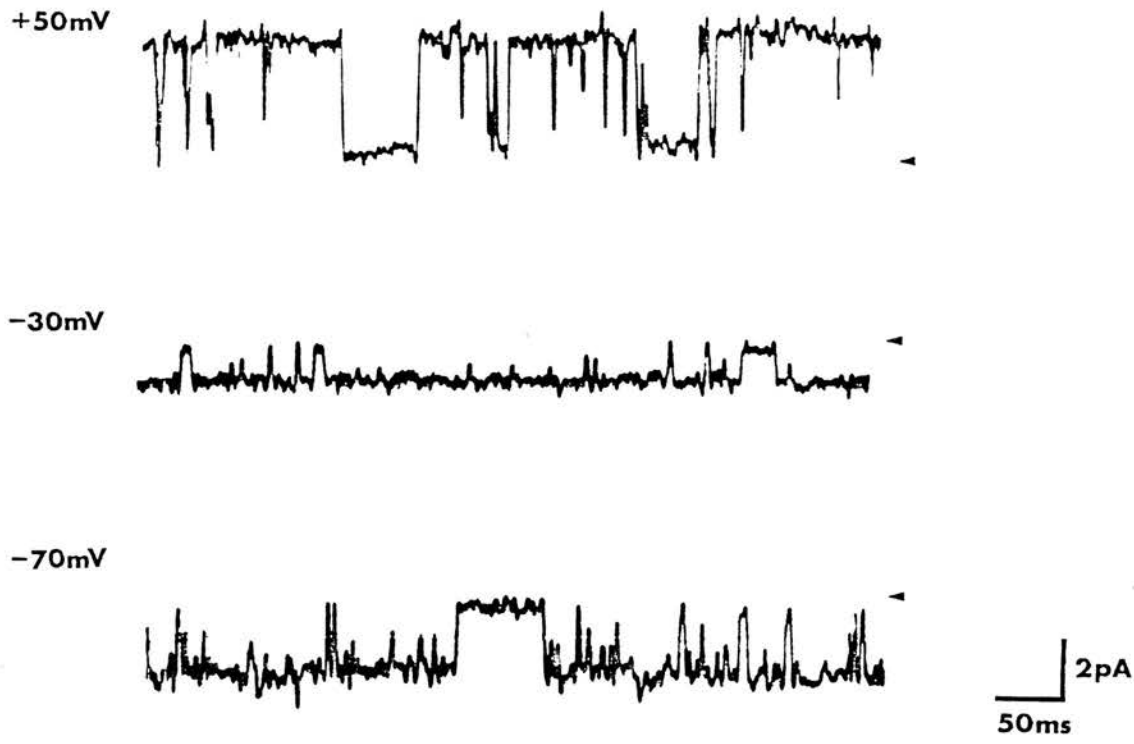
The P_o was plotted against membrane potential for this channel, and shows little voltage-dependence over the membrane potential range +60 to -80 mV (Fig.8.9B). This effect of ADP was time-dependent; little change in the P_o was measured soon after addition of ADP (5 min), while after a long exposure to ADP (20 min), the open probability became voltage-independent.

ii. Effect of ADP on the I-V relation of the channel.

It may be that ADP removes some of the rectification seen with channels in the absence of ADP. The single channel current amplitude of a channel in the presence of ADP was not decreased as much as the current amplitude of channels in the absence of ADP at hyperpolarised potentials. The I-V relation of this channel in the presence of ADP is illustrated in Fig.8.10; the conductance at depolarised potentials was 60.2 pS, and 28.4 pS at hyperpolarised potentials.

Recordings from four patches gave a mean value for the conductance of 64.4 ± 19.6 pS at depolarised potentials, similar to that recorded in the absence of ADP. At hyperpolarised levels, the mean value for the conductance was 39.0 ± 10 pS in the presence of ADP; the channel does not exhibit such severe rectification as channels in the absence of ADP.

A



B

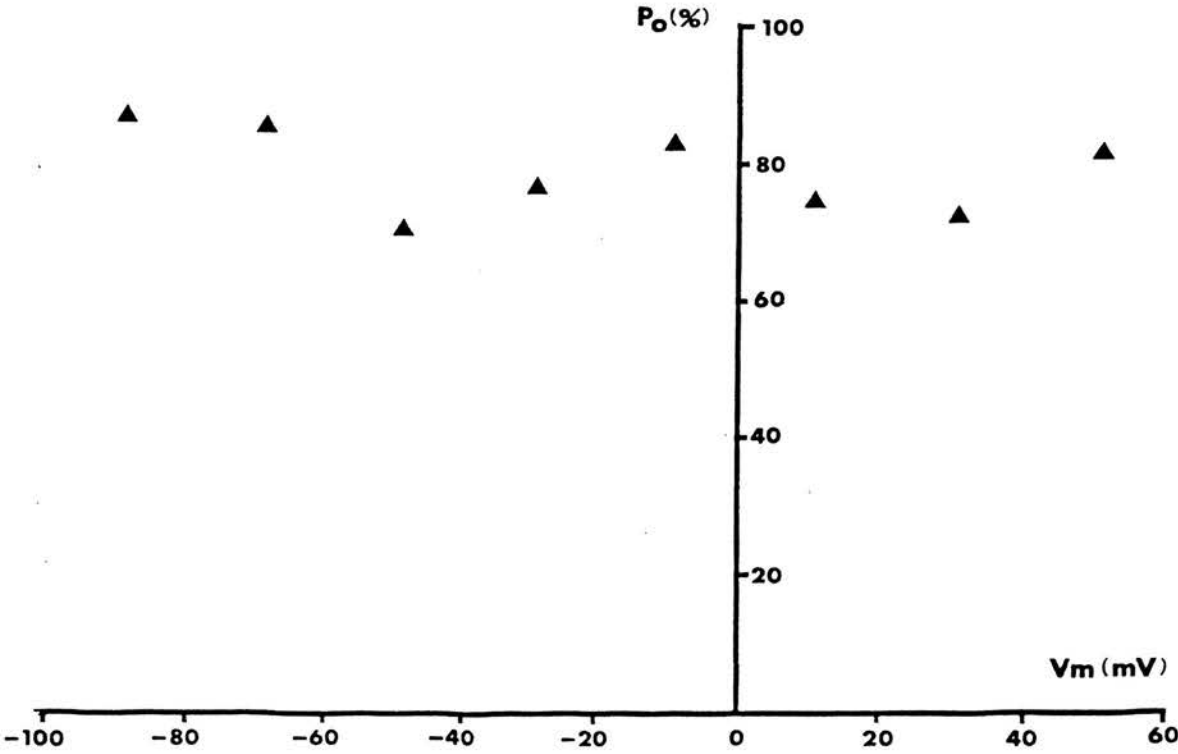


Figure 8.9. Effect of ADP on the P_o of a channel in an excised patch of hamster egg membrane. The patch was bathed with symmetrical KCl and $MgCl_2$, with $4\mu M$ Ca^{2+} and 0.1 mM ADP bathing the intracellular face of the patch. **A.** The channel activity was large for all holding potentials from -100 to + 60 mV. The holding potentials are given on the left hand side of the trace. Channel closed states are indicated by the arrows on the right-hand side of the trace. **B.** A plot of membrane potential against P_o for the channel described in A. A decrease in P_o seen in the absence of ADP (Fig.8.2) was not observed here.

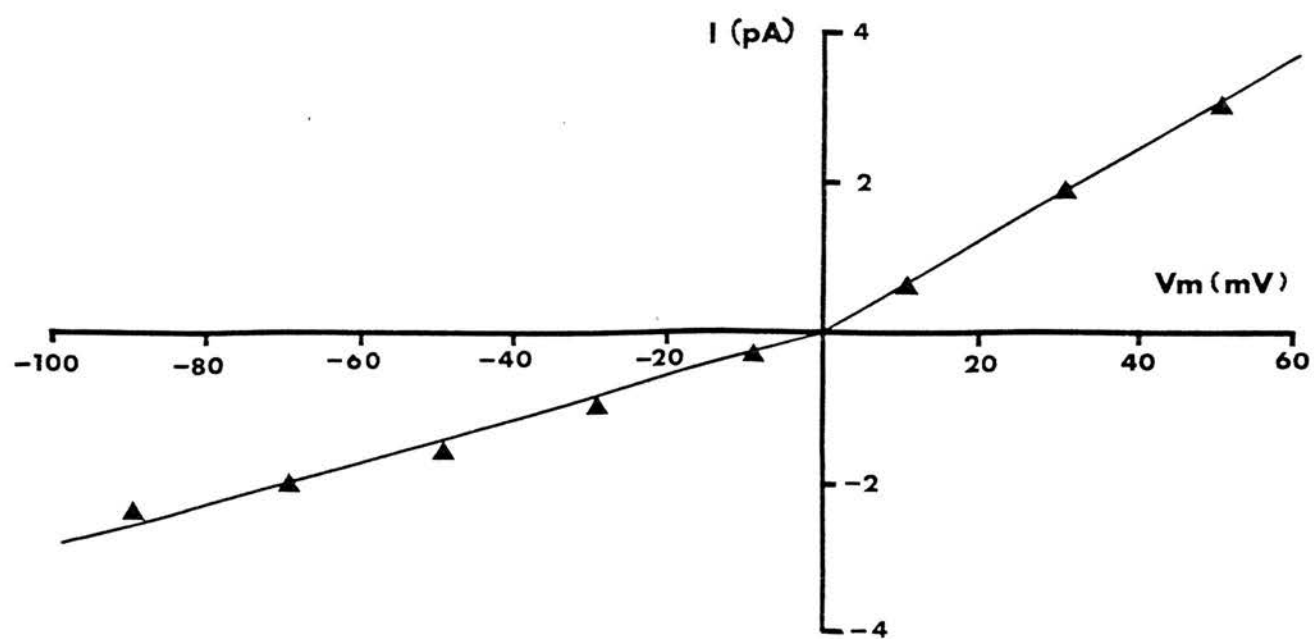


Figure 8.10. The effect of ADP on voltage dependence of single channel conductance of the channel described in Fig.8.11. The I-V relation shown here illustrates the decreased outward rectification in the presence of ADP, compared to the absence of ADP (Fig.8.1B). The patch was bathed with symmetrical KCl and MgCl₂, with 4μM Ca²⁺ and 0.1 mM ADP bathing the intracellular face of the patch.

Discussion.

Ca^{2+} -activated K^+ channels have been reported in many preparations (see Meech, 1978; Petersen and Maruyama, 1984 for review). In the literature, two main types of Ca^{2+} -activated K^+ channel have been distinguished on the basis of unitary conductance size; large conductance channels (180-250 pS with 140 mM KCl on each side of the membrane) and small conductance channels (20 to 50 pS under the same conditions). The channel observed in the hamster egg has novel characteristics, distinguishing it from these previously reported Ca^{2+} -activated K^+ channels.

Comparisons with other Ca^{2+} -activated K^+ channels.

i Voltage dependence.

The channel recorded from the hamster egg has a small conductance and displays outward rectification, with a conductance of about 65 pS at positive potentials and about 10 pS at negative potentials (Fig.8.1B).

However, another interpretation of this I-V relation is that two channels were present in the patch. If this were the case, each channel would require a different voltage sensitivity; one active at depolarised potentials and inactive at hyperpolarised potentials, and the other active at hyperpolarised potentials and inactive at depolarised potentials. It seems unlikely that both of these distinct channels would be observed in all the patches examined so far.

In contrast to the hamster egg channel, small conductance Ca^{2+} -activated K^+ channels from other preparations behave as inward rectifiers, with a conductance of 50 pS and 10 pS at large negative and large positive potentials, respectively (Grygorczyk, Schwarz and Passau, 1984; Sauve, Simoneau, Monette and Roy, 1986). The channel open probability of Ca^{2+} -activated K^+ channels from HeLa cells was voltage-independent between +100 and -100 mV but became larger at membrane potentials more negative than -100 mV (Sauve et al, 1986). Likewise, Grygorczyk and Schwarz (1983) reported an enhancement in channel open probability at hyperpolarised voltages.

Despite the fact that the channel from the hamster egg has a conductance 3-4 times smaller than the large conductance Ca^{2+} -activated channels, they share some features in common. The dependence of the channel open probability of the hamster egg channel (increasing with membrane depolarisation) is compatible with reports on Ca^{2+} -activated K^+ channels from cultured rat muscle (Barrett, et al, 1982), mammalian salivary glands (Maruyama, et al, 1983) and rabbit muscle incorporated into lipid bilayers (Vergara and Latorre, 1983). Additionally, although large conductance K^+ channels are not outward rectifiers, neither do they display inward rectification

(unlike previously reported small conductance Ca^{2+} -activated K^{+} channels), having a linear I-V relation.

In channels from hamster eggs with ADP (0.1 mM) added to the inner membrane surface, outward rectification is attenuated, improving the similarity between these and large conductance Ca^{2+} -activated K^{+} channels.

ii Sensitivity to Ca^{2+} .

The sensitivity of large conductance Ca^{2+} -activated K^{+} channels to $[\text{Ca}^{2+}]_i$ differs from preparation to preparation. In mouse lacrimal glands, activation occurs over the range 0.001 μM to 0.1 μM Ca^{2+} (Findlay, 1984), while in cultured rat muscle, 1.0 μM Ca^{2+} is required for channel activation (Barrett et al, 1983). The sensitivity of the channel from the hamster egg is similar to that of the Ca^{2+} -activated K^{+} channel from HeLa cells (Sauve et al, 1986), where the threshold of activation lies between 0.1 and 0.5 μM (1986).

The decrease in P_o at depolarised levels with high $[\text{Ca}^{2+}]_i$, observed in channels from the hamster egg, has been reported in other preparations. In pancreatic acinar cells, large depolarisations with a $[\text{Ca}^{2+}]_i$ of 0.1 μM led to prolonged states of channel closure (Maruyama et al., 1983b). This is compatible with a slow block of Ca^{2+} on the Ca^{2+} -activated K^{+} channels producing a prolongation of the closed times.

Small conductance Ca^{2+} -activated channels are activated by different ranges of $[\text{Ca}^{2+}]$ from preparation to preparation. In HeLa cells, channel activity was initiated at concentrations from 0.1 to 1 μM (Sauve et al, 1986), while in red blood cells, Ca^{2+} had an effect on P_o over the range 1 to 10 μM (Grygorczyk et al., 1984).

In addition, in HeLa cell channels, it was shown that an increase in cytoplasmic free- Ca^{2+} concentration affects more the number of channel openings per second than the actual channel mean open time.

Dependence of the hamster egg channel on Mg^{2+} .

The success rate of obtaining an active channel was reduced when Mg^{2+} was omitted from the solution bathing the inner surface of the hamster egg membrane. There are reports in the literature of Mg^{2+} enhancing the activity of Ca^{2+} -activated K^{+} channels (Golowasch, Kirkwood and Miller, 1986; Squire and Petersen, 1987).

In channels from rat skeletal muscle reconstituted in lipid bilayers, Mg^{2+} in the range 1-10 mM increases the channel open probability, by increasing the apparent affinity of the channel for

Ca^{2+} and enhancing the sigmoidicity of the Ca^{2+} activation curve (Golowasch et al, 1986). These workers suggest that Mg^{2+} reveals Ca^{2+} binding sites on the protein that are masked in the absence of Mg^{2+} .

In considering the physiological concentrations of Mg^{2+} (estimated to be between 0.4 and 3.0 mM; Corkey, Duszyński, Rich, Matschinsky and Williamson, 1986) the effective concentration range of Mg^{2+} would appear a little high. The effects of Mg^{2+} might have been observed at lower concentrations in the natural plasma membrane; the electroneutrality of the lipids chosen for these artificial membranes may decrease the sensitivity of this channel to divalent cations because of electrostatic surface potential effects (Moczydlowski, Alvarez, Vergara and Latorre, 1985). Confirmation of this idea came from work on salivary acinar cells (Squire and Petersen, 1987) showing a modulation of Ca^{2+} -activated K^{+} channels by lower concentrations of Mg^{2+} . In these experiments, a dose-dependent K^{+} channel activation was obtained with Mg^{2+} in the range 1 mM to 1 μM , at a constant Ca^{2+} concentration of 0.01 μM . Little change was observed by these workers over the range 1 to 10 mM. The concentration of Mg^{2+} used in experiments on the hamster egg was 1.0 mM; although analysis of Mg^{2+} dependence was not performed, low channel activity at 0 mM Mg^{2+} and activity at 1.0 mM Mg^{2+} is compatible with the results from salivary acinar cells.

Modulation of the hamster egg channel by ADP.

The concentration of ADP has been reported to increase by a factor of three after fertilisation (Leese, Biggers, Mroz and Lechene, 1984). It may be that this nucleotide acts as a channel modulator during mammalian fertilisation. The Ca^{2+} -activated K^{+} channel of the hamster egg is sensitive to application of ADP to the intracellular membrane surface. The voltage-dependence of P_o in patches in the absence of ADP was not evident in patches with 0.1 mM ADP bathing the intracellular face of the patch. Additionally, the conductance of channels at negative potentials in the presence of 0.1 mM ADP was larger than in its absence.

Nucleotide-sensitive K^{+} channels have been reported in cardiac ventricular cells (Noma, 1983), pancreatic β -cells (Cook and Hales, 1984) and skeletal muscle (Spruce, Standen and Stanfield, 1985). They are usually not activated by Ca^{2+} ; ^{they are} but inhibited by ^{high Ca^{2+}} the concentrations of Ca^{2+} required ^{to activate} Ca^{2+} -mediated K^{+} channels (Findlay, 1987). ~~In contrast,~~ ^{the} the channel from the hamster egg is activated by both Ca^{2+} and ADP.

Interestingly, the inhibition of nucleotide-sensitive K^{+} channels by ATP has been reversed by application of ADP in an insulin-secreting cell line (Dunne and Petersen, 1986) using the open cell-attached configuration. This method reduces the run-down of activity channel activity observed using excised patches (Kakei, Kelly, Ashcroft and Ashcroft, 1986). The latter group demonstrated a

similar reversal of ATP inhibition of K⁺ channels by ADP in rat pancreatic B-cells. When they excised a patch of membrane into a solution containing 4 mM ATP and 1.5 mM ADP, channel activity was unaffected, indicating that this may be the ratio present in the cell under glucose-free conditions. The conclusion of both groups is that the ratio of ADP and ATP regulates channel activity in these channels.

It may be that the activating effects of ADP on the hamster egg channel are due to the relief of an ATP-related inhibition of channel activity. The fact that some patches were inactive until 20 min after excision suggests that some intracellular factor inhibits the activity of these channels in the unfertilised egg; this factor may be ATP.

Subconductance states.

Looking at the acetylcholine (ACh) receptor of embryonic rat muscle, Hamill and Sakmann (1981) found a second subconductance state, for each of the two characteristic main states of the ACh channel. In each case, the pattern of channel opening was such that once a main open state was reached, the channel either shut completely or the current dropped to the sublevel; also, they never observed subconductance channel opening independently. However, since then, reports of substates preceding the main state have been made (Trautman, 1982), as well as openings to the substate occurring independently of openings to the mainstate.

The possibility remains that the states with a different conductance to the main state were due to another Ca²⁺-activated K⁺ channel with different properties. Paramecium are known to possess more than one type of Ca²⁺-activated K⁺ channel (Hinrichsen and Schultz, 1988), as are rat muscle cells in culture (Romey and Lazdunski, 1984).

Physiological significance of the hamster egg channel.

The outward rectification displayed by the channel of the hamster egg may, in part, underly the repetitive hyperpolarisations of the membrane potential observed at fertilisation. Activation of the Ca²⁺-activated K⁺ channel results in K⁺ efflux, causing a hyperpolarisation. When hyperpolarised, the hamster egg channel has a smaller conductance and a lower open probability than at more depolarised potentials. Hence there is a tendency for less K⁺ to leave the cell, and the cell repolarises. Similarly, when the cell depolarises, the channel conductance and P_o increases to produce K⁺ efflux, hyperpolarising the cell. This may therefore act as a servo-mechanism to prevent large swings in membrane potential. The time-course of each repetitive Ca²⁺ rise at fertilisation is greater than the duration of the hyperpolarisation it evokes (Igusa and Miyazaki,

1986); the outward rectification of these Ca^{2+} -activated K^+ channels may act to limit the efflux of K^+ , should $[\text{Ca}^{2+}]_i$ be elevated above activating levels for more than an optimum period.

SECTION 9 General discussion.

In this study, five methods of activating Ca^{2+} -activated K^{+} channels were applied, each involving a rise in intracellular Ca^{2+} ; iontophoretic Ca^{2+} injection, application of high pH solution, sperm-activation, action potential stimulation and direct application of Ca^{2+} to an inside-out membrane patch. These methods have given information on several characteristics of the Ca^{2+} -activated K^{+} channel; firstly, the interaction between Ca^{2+} efflux from the hamster egg and activation of the Ca^{2+} -activated K^{+} channel, secondly, a possible role for a system amplifying $[\text{Ca}^{2+}]_i$, and thirdly, the behaviour of the channel at the single-channel level. The relevance of these results to fertilisation in the hamster is discussed.

The Ca^{2+} -activated K^{+} channel; its relation to Ca^{2+} influx and Ca^{2+} extrusion across the plasma membrane of unfertilised hamster eggs.

A Ca^{2+} -activated K^{+} conductance underlies the repetitive hyperpolarisations recorded in the hamster egg at fertilisation (Miyazaki and Igusa, 1982). The activation of the channels responsible reflects transient rises in the intracellular Ca^{2+} concentration (Miyazaki, Hashimoto, Yoshimoto, Kishimoto, Igusa and Hiramoto, 1986). Similarly, oscillations in intracellular Ca^{2+} have also been measured in fertilised mouse eggs (Cuthbertson and Cobbold, 1985) and hormone-stimulated rat hepatocytes (Woods, Roy, Cuthbertson and Cobbold, 1986), using the photo-sensitive agent aequorin. Oscillations of membrane potential resulting from activation of Ca^{2+} -activated K^{+} channels have been reported in several other preparations under a variety of conditions; caffeine-treated sympathetic ganglion neurones (Kuba, 1980), spontaneously-activated fibroblastic L-cells (Okada, Tsuchiya and Yada, 1982; Ueda, Oiki and Okada, 1986), and histamine-treated HeLa cancer cells (Sauve, Simoneau, Parent, Monette and Roy, 1987).

The results of my study relating to the inhibition of the Ca^{2+} extrusion system of the hamster egg are interesting in the light of the model proposed by Okada, Tsuchiya and Yada (1982) for the oscillatory responses in fibroblastic L-cells. In their scheme, the stimulus for oscillation produces an influx of Ca^{2+} through Ca^{2+} channels, leading to a rise in intracellular free Ca^{2+} . This activates the Ca^{2+} -activated K^{+} channels, producing a hyperpolarisation of the membrane. The rise in $[\text{Ca}^{2+}]_i$ then initiates Ca^{2+} extrusion from the cell via a Ca^{2+} pump on the plasma membrane. Thus the intracellular Ca^{2+} oscillations are mediated by a feedback system between Ca^{2+} channels (influx) and a Ca^{2+} pump (efflux).

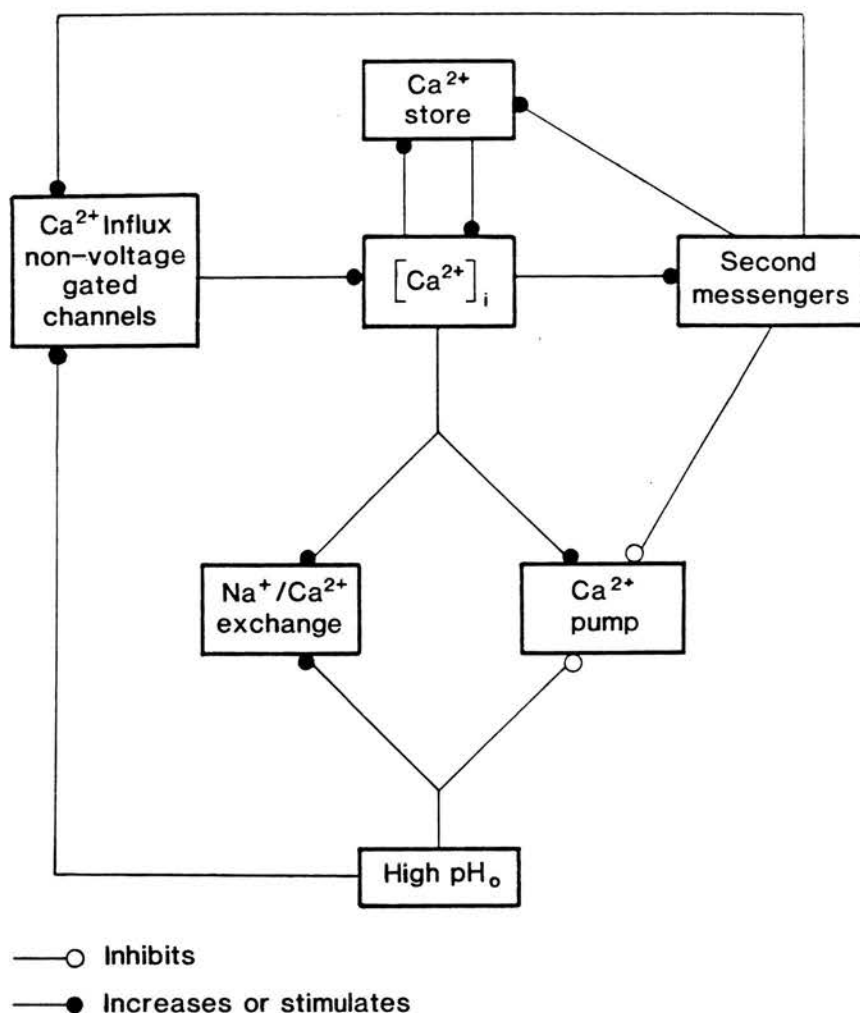


Figure 9.1. Diagram of possible mechanisms involved in regulation of the intracellular free calcium concentration. The calcium dependent hyperpolarisation induced by solutions of high pH can be ascribed to inhibition of the calcium pump along with a possible stimulation of calcium influx through non-voltage-gated calcium channels. The resulting rise in the intracellular free calcium concentration could be amplified through a system involving an intracellular store and the production of second messengers. The transient increases in the intracellular free calcium concentration, observed at fertilisation in the hamster egg may also be mediated by some of the systems in this diagram, leading to the activation of calcium-activated potassium channels.

Raising pH may produce its effect on a similar system, where inhibition of the Ca^{2+} pump of the hamster egg membrane (Georgiou et al, 1988) leads to a rise in intracellular Ca^{2+} , activating Ca^{2+} -mediated K^{+} channels, hyperpolarising the membrane (Fig.9.1). The dependence of the response to high pH_i on external Ca^{2+} may reflect an enhancement of Ca^{2+} influx through non-voltage gated Ca^{2+} channels, similar to that reported for the L-cells (Fig.9.1). Other voltage-insensitive Ca^{2+} channels have been reported in T-cells (Kuno, Goronzy, Weyand and Gardner, 1986).

The Ca^{2+} -activated K^{+} channel and its relation to intracellular Ca^{2+} stores and second messenger systems.

It is possible that the proposed pH_o -induced rise of $[\text{Ca}^{2+}]_i$ in the hamster egg is amplified by a number of mechanisms reported in other studies; an initial rise in $[\text{Ca}^{2+}]_i$ due to block of Ca^{2+} extrusion from the hamster egg may be amplified by a Ca^{2+} -induced Ca^{2+} release from intracellular stores. The all-or-none responses to Ca^{2+} injection reported by Igusa and Miyazaki (1983) and the refractory period associated with them was the first piece of evidence that the intracellular Ca^{2+} stores of hamster eggs influence $[\text{Ca}^{2+}]_i$ and subsequent activation of Ca^{2+} -activated K^{+} channels. The delayed hyperpolarisation induced by a single action potential (McNiven, Yoshida, Georgiou and House, 1988) is further evidence that an amplification system can be activated in the hamster egg; the small amount of Ca^{2+} entering the cell via the action potential is insufficient, on its own, to activate enough Ca^{2+} -activated K^{+} channels to produce a hyperpolarisation. The proposed mechanism for this is illustrated in Fig.9.1. Attempts to stimulate release of Ca^{2+} from the Ca^{2+} store of the hamster egg using caffeine were unsuccessful.

Similarly, Okada, et al (1982), could find no evidence of an involvement of Ca^{2+} stores in the hyperpolarisations of the fibroblastic L-cells, using chemicals known to produce Ca^{2+} release from Ca^{2+} stores in other preparations; azide, known to inhibit mitochondrial uptake in fibroblasts, caffeine, dantrolene-Na and oxalate, which affect microsomal Ca^{2+} transport had no effect on the hyperpolarisations. However, these are negative results, and Henkart and Nelson (1979) have shown that the responses of L-cells can occur in the absence of extracellular Ca^{2+} , suggesting an important role for Ca^{2+} stores in sustaining these responses. In addition, these workers have produced electron micrographs of a potential store of releasable Ca^{2+} (Nelson and Henkart, 1979). Evidence for a cytoplasmic store, sensitive to IP_3 , has recently been provided for non-muscle cells, from cell-lines of rat pancreas and liver (Volpe, Krause, Hashimoto, Zorzato, Pozzan, Meldolesi and Lew, 1988).

In addition to the possibility of Ca^{2+} -induced Ca^{2+} release acting as an amplification system, a rise in $[\text{Ca}^{2+}]_i$ may promote the production of a second messenger such as inositol trisphosphate

(IP₃). This second messenger has been shown to cause Ca²⁺ release from stores in a number of preparations including sea urchin eggs (Whitaker and Aitchison, 1985) and frog eggs (Parker and Miledi, 1987). The sea urchin egg has proved to be a useful system for investigating second messengers, as it is possible to microinject it with specific inositol phosphates (Irvine and Moor, 1986). When the appropriate second messengers are injected a fertilisation envelope is raised, due to a rise in [Ca²⁺]_i. There are two components leading to this rise in [Ca²⁺]_i; one is a direct release of Ca²⁺ from intracellular stores (via IP₃), the second is subsequent to this and involves metabolism of IP₃ to inositol tetrakisphosphate (IP₄). Irvine and Moor propose that IP₄ opens non-voltage gated Ca²⁺ channels in the plasma membrane, leading to an influx of Ca²⁺. Both these events are required for activation of the egg to occur. Recently, a similar effect was seen in acinar cells (Morris, Gallacher, Irvine and Petersen, 1988) showing a synergistic effect of IP₃ and IP₄ in sustaining a hyperpolarisation mediated by a Ca²⁺-activated K⁺ channel. Considering the size of the sperm, the amount of injected Ca²⁺ or IP₃ required to raise the fertilisation membrane of sea urchin eggs is rather large. The initial mobilisation of IP₃ is possibly mediated by a factor recently isolated from sea urchin sperm (Iwasa, Russell, Ehrenstein and deFelice, 1988). This is a heat-stable, trypsin-sensitive protein with a 15 kD molecular weight, which when injected into sea urchin eggs raises the fertilisation membrane. Injection of IP₃ into an unfertilised hamster egg produced a transient rise in [Ca²⁺]_i (Miyazaki, 1987). When GTPγS, a hydrolysis-resistant analogue of GTP was injected, however, periodic rises in [Ca²⁺]_i, similar to those induced by sperm were recorded. Sperm-induced rises in Ca²⁺ were inhibited by GDPβS, a hydrolysis-resistant analogue of GDP. Similarly, results have been reported for the activation of *Xenopus laevis* eggs by GTPγS (Kline and Jaffe, 1985). This would suggest that sperm-egg fusion activates phospholipase C via a GTP-binding protein in the membrane. This leads to hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce IP₃ and so mobilises Ca²⁺ ions from intracellular stores. Bearing in mind the effects of pH_o on the electrical properties of the hamster egg, it is possible that other factors may be involved in the amplification system. For example, the IP₃ produced by sperm-egg fusion might inhibit Ca²⁺ extrusion, as has been reported for vascular smooth muscle (Popescu, Hinescu, Musat, Ionescu and Pistritzu, 1986). Another possibility is that IP₃ might directly activate non-voltage gated Ca²⁺ channels and thus augment Ca²⁺ influx as observed in T-lymphocytes (Kuno and Gardner, 1987). These additional possibilities for the amplification system of the hamster egg have been included in Fig.9.1.

Several other possibilities exist for the pH_o effect that have not been included in Fig.9.1. Firstly, the rise in pH_o may directly affect the plasma membrane, stimulating the hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce IP₃, and thus contribute to the rise in [Ca²⁺]_i. There does not seem to be any literature to support this hypothesis, though. Secondly, high pH_o may have a direct effect on Ca²⁺-activated K⁺ channels enhancing their activity. There is evidence

in the literature of high pH (9.0), in conjunction with high $[Ca^{2+}]_o$, producing an increase in the channel open probability of Ca^{2+} -activated K^+ channels in HeLa cells (Simoneau, Sauve and Roy, 1984). However, there are also reports in the literature of raised pH_o having no effect on the activity of Ca^{2+} -activated K^+ channels (Light et al, 1987). Analysis at the single channel level will resolve whether high pH affects the activity of Ca^{2+} -activated K^+ channels in the hamster egg or not.

Properties of the Ca^{2+} -activated K^+ channel at the single channel level.

Investigation of the Ca^{2+} -activated K^+ channel of the hamster egg using macroscopic (intracellular and voltage clamp) recording techniques have revealed a channel that was similar those previously reported. However, at the single channel level, it appears to have some unusual properties when compared to the same channels in other preparations. From its conductance, it fits into the category of the small conductance Ca^{2+} -activated K^+ channel. However, its properties as an outward rectifier contrast it to other small conductance Ca^{2+} -activated K^+ channels, which tend to behave as inward rectifiers. Additionally, the channel open probability increases with membrane depolarisation; this is a characteristic of large conductance Ca^{2+} -activated K^+ channels. Small conductance channels are usually voltage independent over the range -50 to +50 mV (Sauve et al., 1986). The effects of Mg^{2+} on activity have previously been reported, but the removal of voltage dependence of a Ca^{2+} -activated K^+ channel by ADP is a new finding.

There are reports in the literature of single channel recordings of bursts of activity of Ca^{2+} -activated K^+ channels underlying the hyperpolarising responses of HeLa cancer cells (Sauve, Simoneau, Parent, Monette and Roy, 1987) and human macrophages (Ince, Van Duijn, Ypey, Van Bavel, Weidema and Leijh, 1987). It would be interesting to record Ca^{2+} -activated K^+ channels from the hamster egg in the cell-attached configuration during fertilisation, a profile of activity would be obtained against which the effects of various nucleotides on excised patches could be compared.

Significance of the results to fertilisation in the hamster.

In unfertilised hamster eggs, it was shown that treatments capable of inhibiting Ca^{2+} extrusion could induce membrane hyperpolarisations under certain conditions. Fertilised eggs appeared to be even more sensitive to these treatments. Inhibition of Ca^{2+} extrusion may therefore be a contribution to the repetitive hyperpolarisations observed at fertilisation. Considering the mild alkalinity reported for mammalian oviducal fluid, it seems reasonable to suppose that the inhibition of Ca^{2+} extrusion at a raised pH_o in hamster eggs is of physiological significance. The

sensitivity of fertilised eggs to manipulations of intracellular Ca^{2+} by iontophoretic Ca^{2+} injection has been reported by Igusa and Miyazaki (1983); the sensitivity to Ca^{2+} is 10-fold higher in fertilised compared to unfertilised eggs. Given the results of single channel analysis indicating an enhancing effect of ADP on these channels, and the rise in ADP reported in fertilised mouse eggs (Leese et al, 1984), the Ca^{2+} -activated K^{+} channels of the hamster may be more sensitive to Ca^{2+} because of an increase in the ADP concentration. It is possible also that Mg^{2+} may have an influence on the activity of the channels at fertilisation; unfortunately there is no information in the literature to date on the intracellular Mg^{2+} concentrations of eggs before or after fertilisation.

It may be that the model depicting the possible factors involved in the pH_o -induced hyperpolarisation (Fig.9.1) also represents some of the factors responsible for the events seen at fertilisation.

Possible role for the Ca^{2+} -activated K^{+} channel in the hamster egg.

The discovery of voltage-gated Ca^{2+} channels in the hamster egg (Miyazaki and Igusa, 1981) might, on first appearances, suggest a relation between this channel and the Ca^{2+} -activated K^{+} channel in maintaining membrane potential, similar to that ascribed to endocrine cells (Petersen and Maruyama, 1984). However, the voltage-gated K^{+} channels of the hamster egg are inactivated at the recorded resting potential of the egg, suggesting a role of this sort is unlikely.

The repetitive hyperpolarisations of macrophages have been implicated in the process of phagocytosis (Kouri, Noa, Diaz and Niubo, 1980) and chemotaxis (Gallin and Gallin, 1977). Although it is not clear what the implications of chemotaxis in the egg would be, incorporation of the sperm by the egg could be considered as a form of phagocytosis. However, it seems likely that the hyperpolarisations in macrophages and hamster eggs merely reflect the transient rises in Ca^{2+} that accompany the various events in each preparation; in macrophages, it is the rise in intracellular Ca^{2+} rather than the changes in the membrane potential that determine phagocytotic activity (Okada, Tsuchiya, Yada, Yana and Yawo, 1981). In hamster eggs, it is probably the transient rises in $[\text{Ca}^{2+}]_i$ that are important in fertilisation; a rise in $[\text{Ca}^{2+}]_i$ is crucial in most eggs to promote fertilisation and parthenogenetic activation. However, a possible role for Ca^{2+} activated K^{+} channels in modulating the $[\text{K}^{+}]_i$ and thus some physiological function, such as regulation of metabolic activity, cannot be ruled out; significant changes in $[\text{K}^{+}]_i$ accompanying oscillations in $[\text{Ca}^{2+}]_i$ were measured in fused fibroblastic L cells (Ueda, Oiki and Okada, 1986).

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